Effect of In vitro Culture of Human Myometrial and Fibroid Smooth Muscle Cells on Protein Estrogen Receptor

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Abstract

Background: Uterine leiomyomas (fibroids, myomas) are the most common benign smooth muscle cell tumors in the reproductive tract. The sex hormone estrogen has been implicated as a major regulator of fibroid growth. Estrogen exerts its physiological effects on the target cells by binding to specific nuclear receptors, the estrogen receptor α (ERα) and estrogen receptor β (ERβ).

Objective: To assess the expression of ERα and progesterone receptor (PR) on primary culture smooth muscle cells (SMCs) from myometrial and fibroid tissue for the potential use of primary culture SMCs as an experimental model for studying ER activity, PR activity, and drug testing.

Methods: Quantitative real-time PCR (qPCR) and Western blot analysis were used to compare ERα between paired myometrial and fibroid tissues, as well as cells isolated from the same tissues and cultured up to the three passages.

Results: ERα and PR levels in cultured cells were rapidly lost comparing with whole tissues for both myometrium and fibroid. Moreover, ERα protein was degraded after tissue digestion prior to the culturing process.

Conclusions: Primary human myometrial and fibroid smooth muscle cell cultures are not suitable for studying ER in vitro model. The stable ER and PR cell lines were the model of choice.

Keywords: Leiomyoma, fibroid, myometrium, estrogen receptor, in vitro culture.

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Introduction

Uterine leiomyoma or fibroid is a benign tumor arising from smooth muscle cells in the uterus. The fibroid is commonly found in the reproductive women age between 20 and 50 years\(^{(1)}\), up to 70% of cumulative incidence in menopausal women\(^{(2)}\). Common symptoms of uterine leiomyoma include menorrhagia, miscarriage, difficult pregnancy, frequent urination and constipation. About one-third of women diagnosed with uterine leiomyoma are symptomatic; hence, the fibroid often requires surgical removal. The incidence of uterine leiomyoma in Thai population has never been reported. The exact cause of uterine leiomyoma is still unclear but some studies have reported that the enlargement of fibroid is regulated by estrogen\(^{(3-5)}\). Estrogen activates estrogen receptor (ER) presenting on the nuclear membrane which then translocating into the nucleus to act as a transcription factor. Previous study showed that the expression of ER in uterine leiomyoma is higher than the normal myometrium\(^{(6,7)}\). In addition, clinical evidence also shows a relationship between level of estrogen and fibroid growth. Most fibroids increase in size among women in the reproductive age that associated with a high level of estrogen, and decrease in size after oophorectomy or menopause.

ER is classified into two classes: NR3A1 (ER\(\alpha\)) and NR3A2 (ER\(\beta\)). ER\(\alpha\) plays a role in reproductive system such as endometrium and ovarian stromal cells. ER\(\beta\) acts in the development of brain and cardiovascular system\(^{(8)}\). Previous study demonstrated that mice without \(ESR1\) gene expression encoding for the expression of ER\(\alpha\) failed to naturally reproduce offsprings. On the contrary, \(ESR2\) gene - knock-out mice lacking of ER\(\beta\), but not ER\(\alpha\), can successfully reproduce\(^{(9)}\). A study by Gargett et al concluded that the expression of ER\(\beta\) was not detected in myometrial and fibroid smooth muscle cells (SMCs)\(^{(10)}\), whereas the expression of ER\(\beta\) in fibroid microvascular endothelium was detected\(^{(11)}\).

The focus of this study is to assess the expression of ER\(\alpha\) on primary culture SMCs for the potential use of primary culture SMCs as an experimental model for studying ER activity or drug testing. To date, human myometrial and fibroid SMC stable cell lines have not been well established; therefore, we tried to develop primary cell culture of myometrial and fibroid SMCs as a model for studying the development of fibroids as well as for ER and PR study. The aim of this experiment is to compare the expression of ER\(\alpha\) between myometrial and fibroid tissues collected from patients during culture, before, and after digesting tissue using Western blot analysis and qPCR.

Materials and methods

Tissue collection

Fibroid and myometrial tissues were obtained from five pre-menopausal women age 30 - 40 years who underwent hysterectomy for uterine fibroids (Figure 1) and had not received hormonal or drug therapy. Informed consent was obtained. Ethical approval was obtained from the Human Rights and Ethic Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University. Tissue was snap frozen in liquid nitrogen immediately after excision, and was also collected in the Hank buffer saline solution (HBSS) (Gibco BRL) during transporting to the laboratory in order to processing and culturing. Snap frozen tissue was stored at \(-80\) °C until protein extraction.

Primary cell cultures

The tissue specimen were minced into small pieces and digested with collagenase type II (Worthing-
Fresh operative specimens of a uterus were obtained from women in the proliferative phase and secretory phase of menstrual underwent elective hysterectomies.

In the culture medium (phenol red-free DMEM/F12) (Gibco BRL) at 37 °C for 3-4 hours. The digested tissues were processed through the cell strainer. The cells were then collected by centrifugation at 1,500 rpm for 10 minutes and washed several times with phosphate buffer solution (PBS). The resulting cell pellets were resuspended in the culture medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and antibiotic solution (1x10^5 U/L penicillin and 50 mg/L streptomycin). The cells were seeded in 75 cm² culture flask. The experiments were done on secondary and tertiary cultures. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Growing cells were assessed in the experiments.

Protein extraction and Western immunoblotting

The homogenized tissue and cultured cells were lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail tablets), and incubated at 4 °C for 20 minutes. The extracts were centrifuged at 14,000 xg for 15 minutes and the supernatant were then collected. Protein concentrations were determined by the Bradford assay and equal amount of total protein were loaded into each well. Protein samples (30 μg protein) were resolved by 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk in Tris buffer saline-Tween 20 (TBST) for 1 hour, and incubated overnight with ERα antibody (F10; Santa Cruz Biotechnology, Santa cruz, USA) and GAPDH antibody as control (6C5; Santa Cruz Biotechnology, Santa cruz, USA) at a 1:200 dilution in 5% blocking solution (TBST that contained 5% non-fat dry milk) at 4 °C on a shaker. After being washed three times in TBST, the membranes were incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa cruz, USA) at 1:2,000 dilution in 5% blocking buffer for 1 hour. The antigen-antibody complexes were detected with the enhanced chemiluminescence detection system (Thermo Scientific, USA) and images were acquired by C-DiGit Blot Scanner (Licor Biotechnology, USA).

Reverse transcription and qPCR

Total RNA from homogenized tissue and culture cells was extracted by RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instruc-
tions. Reverse transcription reactions were carried out using the reverse transcriptase system (Promega, Madison, WI). All qPCR were performed by a real-time PCR machine CFX96 (Bio-Rad Laboratories) with the SoFast EvaGreen (Biorad) and gene-specific primers purchased from Integrated DNA technologies (IDT). Quantification of gene expression was assessed by the comparative cycle threshold \( (2^{-\Delta\Delta CT}) \) method. The following primer pairs were used for amplification:

ESR1 forward, 5'-TGATGAAAGGTGGGATACGAA3'; ESR1 reverse, 5'-AAGGTTGGCAGCTCTCAGGTGT3';

GAPDH forward, 5'-TCTCTGCTCCTGTGT3';

GAPDH reverse, 5'-ACCAAATCCGGTGTGATG3';

TFF1 forward, 5' -GCCCTCCCAGTGTGAAATA3';

GAPDH reverse, 5' -CTGGAGGGACGTCAGGTA3';

V-MYC forward, 5' -TGCCCTGCTGTTGG3';

GAPDH reverse, 5' -GTCTCAGTGGTGTAAG3'.

Results

**ERα protein expression in the primary culture of smooth muscle cells (SMCs)**

ERα proteins (66 kilodaltons) from myometrial and fibroid tissues obtained from the same patient before and after digestion with enzyme collagenase type II and the ones from different culture passages were compared by Western blot analysis. With the same concentration of protein used, the expression of ERα was observed in both types of tissues prior to digestion. However, after digestion with collagenase type II the expression of ERα was not observed from any passage (Figure 2). On the contrary, ERα expression of fibroid SMCs was noticed in P0 but not in the later passage (Figure 2). The digestion has no effect on Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression on both myometrial and fibroid SMCs.

![Figure 2](image.png)

**Figure 2** Effects of sex steroids on the expression of ERα protein (66 kilodaltons) assessed by Western blot analysis in SMCs culture in vitro compared with the whole tissues both myometrial and fibroid. GAPDH (37 kilodaltons) was used as a protein load control.

The expression of ER was observed in both types of tissues prior to digestion (Lane 1 and 5). After digestion with collagenase type II the expression of ERα was not observed from any passage (Lane 2-4). ERα expression of fibroid SMCs was noticed in P0 (Lane 6) but not in the later passage (Lane 7-8).
**ERα and PR gene expression in the primary culture of SMCs and fibroid and myometrial tissues**

The qPCR analysis of *ESR1* gene between myometrial and fibroid tissues and their respective cells. P0 to P3, showed loss of gene expression in both myometrial cells and fibroid SMCs after the digestion process (P0-P3) (Figure 3). The expression of *ESR1* gene was found to be 2.7 fold higher in fibroid tissue when compared to myometrial tissue using $2^{ΔΔCt}$ method (Figure 4). Loss of *PR* gene expression was demonstrated in both myometrial cells and fibroid SMCs after the digestion (Figure 5). Slightly decreased *avian myelocytomatosis viral oncogene neuroblastoma derived homolog (V-MYC)* without decreased *trefoil factor 1 (TFF)-1* gene expression in both myometrial cells and fibroid SMCs was demonstrated (Figure 6). Both genes were estrogen-regulated target genes.

**Figure 3** ESR1 gene expression in myometrial tissue and myometrial cell culture (A) and in fibroid tissue and fibroid SMCs culture (B). After the digestion process (P0-P3), gene expression was lost in both myometrial and fibroid SMCs culture.

**Figure 4** Comparing of ESR1 expression level between myometrial and fibroid tissues. The ESR1 gene expression in fibroid tissue was 2.7 fold higher than myometrial tissue.
Figure 5 Comparing of PR expression levels between myometrial and myometrial cells, and fibroid tissues and fibroid SMCs. PR gene expression was lost in both myometrial cells and fibroid SMCs after the digestion.

Figure 6 Comparing of V-MYC (A) and TFF-1 (B) expression levels between myometrial and myometrial cells, and fibroid tissues and fibroid SMCs.

Discussion

In the present study we cultured SMCs extracted from the uterine fibroids and adjacent normal myometrial tissues at least 3 passages, and performed gene and protein expression for SMCs and the myometrial tissues. We found that SMCs from uterine fibroids grew slowly in the culture and became senescent earlier than the ones from the normal myometrium. The expression of ER was reduced significantly when the tissues were digested with enzyme collagenase type II. Furthermore, the expression of the receptors was also decreased during the culture.
of 3 passages. The expression of estrogen targeted genes, i.e. V-MYC and TFF-1, was slightly decreased. We had also studied different methods, for example, using tissues from younger patients, modifying cell culture conditions, but the results showed no expression of ER and PR at both the mRNA and the protein levels which was consistent to previous studies\(^1\). However, Sadovsky et al\(^{12}\) studied the expression of ER from SMCs using rabbit model where as Severino et al\(^{13}\) isolated SMCs from uterine fibroids and myometrium using tissue minced explant method, not enzyme digestion. Zaitseva et al\(^{14}\) isolated SMCs using relatively similar technique to ours, but the authors digested the uterine fibroids and myometrium with trypsin. They also showed the reduction of ER\(\alpha\) and PR in the cell culture of up to 3 passages when incubated with \(\beta\)-estradiol (E\(_2\)) and progesterone. Global gene expression with microarray hybridization, and RT-PCR was performed and found that there were 648 genes changed between tissues and cultured cells. ER\(\alpha\) was one of 648 genes that found down-regulation. Therefore, the decreased ER\(\alpha\) of primary culture of SMCs was not depend on SMC isolation technique, enzyme digestion or tissue explants, and the changes could occur in other species, not just human. We speculate that the characteristics of SMCs would be different when the cells were separated from each other versus when the cells were organized in the tissues. Further studies are needed to identify the cause of these changes, for example, the interaction among the SMCs that enhances the behavior of these cells.

Role of estrogen on the reduction of ER\(\alpha\) expression in primary SMCs is questionable. Severino et al\(^{13}\) reported a loss of ER and PR 72 hours after the incubation with estradiol (E\(_2\)) in leiomyoma and myometrial explants culture. Their result was in line with our finding. Primary cell culture in our experiment was also treated with hormone but showed no expression of ER\(\alpha\) receptors (data not shown). From literature review, only few studies reported the mechanism of the change in ER level by using E\(_2\) as a stimulant. Alarid et al\(^{15,16}\) reported a clear decreased level of ER protein 1 hour after treatment with estrogen, and the basal half-life of ER\(\alpha\) was between 3-5 hours depending on cell types. On the other hand, Valley et al demonstrated that the acute E\(_2\) administration shortened the ER\(\alpha\) half-life, while the chronic E\(_2\) treatment prolonged ER\(\alpha\) half-life\(^{17}\). Therefore, the time taken to transport specimen and digestion of specimen need to be considered when conducting the study regarding these issues.

Both mRNA and protein level analysis showed the expression of ER\(\alpha\) in fibroid tissue was higher than the myometrial tissue. Previous study reported that steroid hormones produced from ovary, especially estrogen and progesterone, play important roles in the development of uterine tumor\(^{18}\). We did not study ER\(\beta\) expression because SMCs do not express this receptor\(^{10}\). Progesterone has dual effect on growth of tumor, both stimulation and suppression. On the one hand, the stimulation of tumor growth by progesterone mediates through the up regulation of epidermal growth factor and B-cell lymphoma-2 gene involving in the regulation of cells apoptosis especially in malignancy cells, but on the other hand, progesterone also exerts down regulation of cytokines tumor necrotic factor-\(\alpha\) and insulin-like growth factor-1 controlling the growth of cells\(^{19}\). Therefore, the role of progesterone on tumor growth is still unclear. Comparison of ER expression between normal myometrial and fibroid tissue shows a higher expression of ER in fibroid than the myometrial tissue in the form of \(\alpha\) rather than \(\beta\)\(^{7,20,21}\).

Since the rapid loss of ER\(\alpha\) and PR from the primary cell culture of myometrium and uterine
fibroid, in order to study the behavior of SMCs related to estrogen and progesterone, an experimental model which contains stable ER$\alpha$ and PR is needed. Many studies using primary cell cultures from myometrial and fibroid SMCs transfected with adenovirus/ER$\alpha$ for maintaining the steroid receptors-positive primary cultures as a model for studying the expression level of ER have been conducted$^{[22-24]}$. Another study could show an increase in ER$\alpha$ in both myometrial and fibroid SMCs after treatment with $17\beta$-estradiol$^{[26]}$. In addition, immortalization of fibroid SMC line by using retroviral vector containing human telomerase reverse transcriptase (hTERT) to induce telomerase activity or organotypic culture can be used to maintain in vivo environment in the study$^{[26,27]}$.

In conclusion, using primary cell cultures of myometrial and fibroid SMCs as a model for study of effect and mechanism of estrogen analog molecule showed the disappearance of ER and PR during culture and digestion. Therefore, for further study, the immortalized cell line such as estrogen stimulated Ishikawa cells, a human Asian endometrial adenocarcinoma, should be used to maintain the level of ER$\alpha$. Moreover, clinical study can be conducted to study the effect of estrogen on uterine tumor directly.

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References


ผลการเผาเดี่ยวนิสิทธิ์กล้ามเนื้อเรียบบางกล้ามเนื้อหลักและความต้านทานเป็นวิธีออกตัว

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บทคัดย่อ

ความเป็นมา: เนื่องจากกล้ามเนื้อต้นกล้ามเนื้อหลักซึ่งเป็นกล้ามเนื้อหลักที่ไม่สามารถเกิดจากเชื้อสัตว์เพียงเชื้อเดียวเป็นการเพิ่มจำนวนเซลล์ที่มี discard ไป การเรียบข่ายขนาดของเนื้องอกกล้ามเนื้อหลักของเซลล์ ดัง образомในกล้ามเนื้อหลัก โดยจะออกฤทธิ์ทางด้านการเรียบข่ายขนาดในน้ำมันคลื่นเพื่อควบคุมการทำงานของเซลล์เป้าหมาย หรือ ด้านเรียบข่ายในเซลล์лепโดยได้

วัตถุประสงค์: เพื่อศึกษาผลของการแปลงออกของโปรตีนด้านการเรียบข่ายขนาดเล็กและปล่อยเซลล์ของเซลล์ เพื่อเรียบข่ายมุมมีกล้ามเนื้อจากคนหลุดปกติและเรื้องกล้ามเนื้อ เพื่อใช้เป็นโมเดลในการทำการศึกษาผลต่อไป

วิธีจัด: การศึกษาใช้วิธี Western blot analysis และ Quantitative real-time PCR (qPCR) โดยปรับเทียบเนื้อเยื่อระหว่างเซลล์ลด้านเนื้อเยื่อค้นหลุดหลักและเนื้องอกกล้ามเนื้อหลักจากผู้ป่วยรายเดียวกัน กลุ่มการย่อยเซลล์และหลักการเพาะเลี้ยงเซลล์เป็นจำนวน 3 passages

ผลการศึกษา: มีการสูญหายไปของด้านการเรียบข่ายขนาดและปล่อยเซลล์ในกล้ามเนื้อเรียบหลักและเซลล์จากเนื้องอกกล้ามเนื้อต้นกล้ามเนื้อเรียบหลักและเซลล์จากเนื้องอกกล้ามเนื้อต้นกล้ามเนื้อเรียบหลัก ดังนั้น การใช้เซลล์ที่มีการแปลงออกของด้านเรียบข่าย และปล่อยเซลล์ที่มีหลักจะเหมาะสมกว่า

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