Differential induction of autophagy by mTOR is associated with abnormal apoptosis in ovarian endometriotic cysts

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ABSTRACT: Mammalian target of rapamycin (mTOR) is known to be a major negative regulator of autophagy. Recent studies have shown that mTOR activity is abnormally increased in endometriotic lesions. In endometriosis, abnormal mTOR activity may contribute to the alteration of endometrial cell autophagy, which may affect apoptosis because endometrial cell autophagy is directly involved in the regulation of apoptosis. To test this hypothesis, we investigated whether endometrial cell autophagy is altered by aberrant mTOR activity and is associated with apoptosis in ovarian endometriotic cysts. Our results show that endometrial cell autophagy induction was increased by mTOR inhibition as the menstrual cycle progresses in the normal endometrium, and that it is correlated with apoptosis. However, in endometriotic tissues from ovarian endometriotic cysts, autophagy, mTOR activity and apoptosis were constant throughout the menstrual cycle, suggesting that a constant level of autophagy is maintained by disinhibition of mTOR activity during the menstrual cycle in endometriotic tissues and is related to decreased apoptosis. Indeed, compared with normal endometrium, increased mTOR activity during the secretory phase in endometriotic tissues inhibited autophagy and apoptosis induction. In addition, to determine the direct effect of autophagy induction mediated by mTOR on endometriotic cell apoptosis, endometriotic cells were treated with rapamycin (mTOR inhibitor) with and without 3-methyladenine (3-MA, autophagy inhibitor). Although rapamycin treatment induced autophagy and led to apoptosis promotion, the pro-apoptotic effect of rapamycin was reversed by the addition of 3-MA, suggesting that mTOR inhibition promotes endometriotic cell apoptosis via autophagy induction. In conclusion, our results suggest that aberrant mTOR activity in ovarian endometriotic cysts leads to alteration of endometrial cell autophagy, which is associated with abnormal apoptosis.

Key words: endometriosis / autophagy / mTOR / apoptosis

Introduction

Endometriosis is characterized by the presence of endometrium-like tissue outside the uterine cavity, primarily on the ovaries and pelvic peritoneum. Endometriosis is one of the most common causes of chronic pelvic pain, dysmenorrhea and infertility (Giudice and Kao, 2004; Bulun, 2009) and affects ~5–15% of reproductive-age women and 20–50% of infertile women (Eskenazi and Warner, 1997; Pritts and Taylor, 2003). Various theories have been proposed with regard to the pathogenesis of endometriosis, but there is no consensus on a definitive theory. Accumulated evidence has asserted that reduced apoptosis could contribute to the survival of refluxed endometrial cells at ectopic sites and the establishment of endometriosis (Gebel et al., 1998; Dmowski et al., 2001). Thus, endometriosis is believed to result from reduced apoptosis of endometrial cells. Although this reduction is caused by abnormal expression of apoptosis-related proteins such as BCL2, BAX, Fas and Fas ligand (Harada et al., 1996; Watanabe et al., 1997; Jones et al., 1998; Garcia-Velasco et al., 2002), the exact mechanism that reduces endometrial cell apoptosis in endometriosis has not been fully elucidated.

Autophagy is an intracellular bulk degradation system in which a portion of the cytoplasm is enveloped in double membrane-bound structures called autophagosomes, which undergo maturation and fusion with lysosomes for degradation (Klionsky and Emr, 2000; Levine and Klionsky, 2004). It has also been shown to have a complex relationship with apoptosis in various cells (Gozuacik and Kimchi, 2004; Eisenberg-Lerner et al., 2009). Indeed, several reports have demonstrated that autophagy plays an important role in promoting cell survival by preventing apoptosis (Bauvy et al., 2001; Carew et al., 2010). In contrast, it has been reported that autophagy...
Materials and Methods

Tissue collection

Normal eutopic endometrial tissues (NEETs, n = 20) were obtained from premenopausal women undergoing hysterectomy for uterine leiomyoma or pelvic organ prolapse. Ectopic endometriotic tissues (EETs, n = 25) were obtained from ovarian endometriotic cysts (endometrioma). No study participant had taken oral contraceptives or hormonal agents for at least 3 months before surgery. The average age of participants was 42.3 ± 3.8 years for eutopic endometrial tissues and 30.5 ± 5.3 years for ectopic endometrial tissues. Endometrial tissue samples were divided into five categories according to day of menstrual cycle: early proliferative (days 1–5), mid-to-late proliferative (Days 6–14), early secretory (Days 15–18), mid-secretory (Days 19–23) and late-secretory phase (Days 24–28). Menstrual cycle phase was established based on the patient’s menstrual history. Of the 20 eutopic endometrial samples, four each were early proliferative, late proliferative, early secretory, mid-secretory and late secretory, while 5 each of 25 ectopic endometrial samples were early proliferative, late proliferative, early secretory, mid-secretory and late secretory phase. All samples were snap frozen in liquid nitrogen and stored at −80 °C for western blot analysis. This study was approved by the Ethical Committee of Samsung Medical Center. Written informed consent was obtained from all participants.

In vitro experiments

Subcultured NESCs and ECSCs were seeded at 1 × 10^6 cells/ml in poly-L-lysine-coated non-fluorescent thin-bottom glass culture dishes (MatTek, Ashland, MA, USA). Cells were incubated at 37 °C in 5% CO2 in DMEM/F12 supplemented with 10% FBS, glutamine, HEPES, 100 U/ml penicillin and 100 mg/ml streptomycin. At 70–80% confluence, cultures were treated with serum-free Earle’s Balanced Salt Solution (EBSS) medium (Sigma) for serum starvation. To mimic physiological hormonal changes, cells were also cultured with EBSS medium before hormone treatment. After 24 h of culture, 10 nM estradiol (Sigma) and 1 μM progesterone (Sigma) were added for 24 h and media were replaced with hormone-free media 24 h before analysis. Additionally, ECSCs were cultured in EBSS medium with addition of either 1 μM rapamycin (Sigma) or 1 μM rapamycin + 10 mM 3-methyladenine (3-MA; Sigma) to inhibit mTOR activity and autophagy, respectively. Four hours later, media were removed and stromal cells were collected by scraping to extract proteins for immunofluorescence or electron microscopy. Apoptosis of ECSCs was evaluated using annexin-V/propidium iodide (PI) staining.

Western blot

During autophagy induction, microtubule-associated protein light chain (LC3) is converted from LC3-I to LC3-II. LC3-II then becomes localized to autophagosomes, allows for the reduction in apoptosis found in endometriosis. Therefore, the LC3-II protein was measured by western blot to evaluate autophagy and apoptosis in ECSCs. The LC3-II expression correlates with autophagosome number (Nara et al., 2002). Therefore, the LC3-II protein was measured by western blot to evaluate endometrial cell autophagy. The activity of the mTOR pathway was determined by measuring the phosphorylation of p70S6 kinase (p70S6K) because the p70S6K protein is a direct substrate of mTOR and so its phosphorylation status can be used as an indicator of the activity of the mTOR pathway (Barbassov et al., 2005). Endometrial cell apoptosis was determined by measuring cleaved caspase-3, the final mediator of apoptosis (Porter and Janicke, 1999). In addition, we also evaluated phosphorylation of ribosomal protein S6 kinase (S6K) and the expression of cleaved poly (ADP-ribose) polymerase (PARP) to confirm mTOR activity and apoptosis in rapamycin-treated ECSCs. Endometrial or endometriotic tissues or cultured stromal cells were lysed with ice-cold radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Sigma). For complete solubilization of cellular proteins, lysates were incubated on ice for 30 min, and then...
centrifuged at 13,000 g at 4 °C for 30 min. Whole-cell lysates (20 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA, USA). After blocking with 5% skim milk, membranes were treated with rabbit polyclonal antibodies to LC3 (1:5000; Novus), caspase-3 (1:1000; Cell Signaling), total and phosphorylated p70S6K (thr389) (1:1000; Cell Signaling) or total and phosphorylated S6K (Ser235/236) (1:1000; Cell Signaling) overnight at 4 °C. Membranes were washed three times for 15 min each with washing buffer (PBS containing 0.1% Tween 20) and incubated with an appropriate secondary antibody IgG (1:5000; Santa Cruz Biotechnology) at room temperature for 1 h. After three 15-min washes in washing buffer at room temperature, membrane-bound proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Bands were quantified using NIH Image software (NIH image Processing and Analysis in Java). The expression of LC3-II and cleaved caspase-3 was normalized to β-actin, while the expression of phosphorylated p70S6K and S6K was normalized to total p70S6K and S6K, respectively.

**Immunofluorescence**

Endometrial and endometriotic stromal cells were cultured on sterilized glass coverslips, fixed with 4% paraformaldehyde and blocked with 0.1% bovine serum albumin in PBS. Cells were incubated with anti-LC3 mouse polyclonal antibody (1:500) and cleaved caspase 3 rabbit polyclonal antibody (1:500) in PBS, then with Alexa 488- and 568-conjugated secondary antibodies (1:5000; Vector Laboratories, Burlingame, CA, USA). Slides were mounted in mounting media (Vector Laboratories) with 4',6-diamidino-2-phenylindole (DAPI) for nucleus staining. Images were captured with a confocal microscope (Bio-Rad).

**Transmission electron microscopy**

To identify autophagosomes at the ultrastructural level, endometrial and endometriotic stromal cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 45 min at 4 °C, rinsed in cacodylate buffer, post-fixed in 1% OsO4, in cacodylate buffer, dehydrated and embedded in Eponate. Ultra-thin sections were briefly contrasted with uranyl acetate and photographed with a transmission electron microscope (Hitachi 7100, Tokyo, Japan).

**Assessment of human endometriotic stromal cell apoptosis**

Apoptotic cell percentages were determined with an annexin-V fluorescence isothiocyanate apoptosis detection kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s protocol. Briefly, after drug treatment, 1 × 105 cells were pelleted and washed once with PBS and resuspended in 100 μl binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 5 mM potassium chloride, 1 mM MgCl2, and 2 mM calcium chloride). Subsequently, 5 μl annexin V and PI were added and cells were incubated for 15 min at room temperature in the dark. After incubation, 400 μl binding buffer was added, and cells were analyzed using a FACSaria flow cytometry (BD Biosciences, Heidelberg, Germany). At least 10,000 cells were analyzed per treatment. Data analysis was conducted using CellQuest software.

**Statistical analysis**

Expression levels of all proteins and proportions of apoptotic cells are reported as mean ± standard error. Statistical analysis was performed using ANOVA. Significant differences between the treatment groups were determined by Duncan multiple-range tests. Statistical significance was inferred at P < 0.05.

**Results**

**Autophagy, mTOR activity and apoptosis in NEETs and EETs during the menstrual cycle**

We investigated whether autophagy and mTOR activity are induced differently during the menstrual cycle in the NEETs and EETs from ovarian endometriotic cysts. We measured the expression levels of LC3-II and phosphorylated p70S6K to evaluate autophagy and mTOR activity, respectively. In NEETs, as shown in Figs 1A and B, LC3-II expression increased during the late proliferative phase compared with the early proliferative phase; however, the difference was not significant. LC3-II expression was significantly higher in the early (1.74-fold), mid- (2.57-fold) and late (3.58-fold) secretory endometrium than in the early proliferative endometrium. In contrast, the expression level of phosphorylated p70S6K decreased significantly during the secretory phase compared with early proliferative phase (P < 0.05). Subsequently, we also evaluated the expression of cleaved caspase-3 to examine whether the endometrial cell autophagy mediated by mTOR is associated with apoptosis during the menstrual cycle. In a NEETs, cleaved caspase-3 expression also increased significantly during the secretory phase, peaking during the late secretory phase (P < 0.05; Fig. 1B). In EETs, however, the expression levels of LC3-II, phosphorylated p70S6K and cleaved caspase-3 did not change during the secretory phase and remained constant throughout the menstrual cycle (Fig. 1C). In addition, we compared directly on the same gel the expression levels of LC3-II, phosphorylated p70S6K and cleaved caspase-3 at the late secretory phase of menstrual cycle of NEETs and EET. As shown in Fig. 1D, LC3-II and cleaved caspase-3 expression were significantly lower in the EETs than in the NEETs, while phosphorylated p70S6K was significantly higher (P < 0.05).

**The effects on estrogen and progesterone on autophagy, mTOR activity and apoptosis in NESCs and ECSCs in vitro**

To determine the effect of ovarian steroids on autophagy, mTOR activity and apoptosis of NESCs and ECSCs, we characterized the effects of estrogen and progesterone on LC3-II, phosphorylated p70S6K and cleaved caspase-3 expression in cultured NESCs and ECSCs. Results presented in Fig. 2A and B show that LC3-II expression increased significantly and phosphorylated p70S6K decreased in NESCs cultured with estrogen and progesterone compared with NESCs cultured with estrogen alone (P < 0.05). Following incubation with estrogen and progesterone, removal of both estrogen and progesterone from the media for 24 h also increased LC3-II and decreased phosphorylated p70S6K expression (P < 0.05). Furthermore, the expression levels of cleaved caspase-3 increased significantly in cultured NESCs with the addition of progesterone or withdrawal of both estrogen and progesterone compared with levels of cleaved caspase-3 in NESCs cultured with estrogen alone (P < 0.05). In ECSCs, however, the treatment of estrogen and progesterone failed to change the expression levels of LC3-II, phosphorylated p70S6K and cleaved caspase-3 (Fig. 2C). When comparing NESCs and ECSCs directly on the same gel following removal of estrogen and progesterone, LC3-II and cleaved caspase-3 expression decreased significantly in ECSCs compared with NESCs and phosphorylated p70S6K expression increased (P < 0.05, Fig. 3A).
We also used immunofluorescence to examine the subcellular localization of endogenous LC3 and the expression of endogenous cleaved caspase-3 to evaluate autophagy and apoptosis in NESCs and ECSCs. In NESCs cultured with estrogen alone, a few punctuate LC3-II structures were detected throughout the cytoplasm, with weak staining for cleaved caspase-3 (Fig. 3B, NESCs, top). In contrast, the punctuate LC3-II structures accumulated after withdrawal of estrogen and progesterone, with intense immunoreactivity for cleaved caspase-3 (Fig. 3B, NESCs, bottom). In ECSCs, however, punctuate LC3-II and cleaved caspase-3 staining did not increase with withdrawal of estrogen and progesterone (Fig. 3B, ECSCs, bottom) compared with ECSCs cultured with estrogen only (Fig. 3B, ECSCs, top).

In addition, Fig. 3C shows the transmission electron micrographs of cultured NESCs and ECSCs. Autophagic structures are characterized by multiple autophagosomes, which are double-membraned vacuoles containing engulfed cytoplasmic materials. Some autophagosomes were evident in NESCs cultured with estrogen alone (Fig. 3C, NESCs, top), and the number increased when both estrogen and progesterone were removed from media (Fig. 3C, NESCs, bottom). However, the number of autophagosomes did not increase in cultured ECSCs when estrogen and progesterone were withdrawn (Fig. 3C, ECSCs, bottom) compared with ECSCs cultured with estrogen alone (Fig. 3C, ECSCs, top).

### Apoptosis induced by increased autophagy via mTOR inhibition in ECSCs

To determine whether apoptosis of ECSCs is mediated by autophagy induction by mTOR inhibition, we evaluated the effect of rapamycin, a specific mTOR inhibitor (Schmelzle and Hall, 2000) and 3-MA, an autophagy inhibitor (Backer, 2008) on serum starvation-induced ECSC...
apoptosis. Figure 4A and B showed that rapamycin treatment significantly decreased the expression of phosphorylated p70S6K and S6K compared with ECSCs cultured in serum-starved condition (control); whereas, the expression levels of LC3-II, cleaved caspase-3 and cleaved PARP increased. Rapamycin-stimulated LC3-II expression decreased significantly without phosphorylation of p70S6K and S6K by addition of 3-MA, together with significant decreases in cleaved caspase-3 and cleaved PARP expression ($P < 0.05$). Furthermore, a few punctuate LC3-II structures were present with weak staining for cleaved caspase-3 in the cytoplasm of ECSCs cultured in serum-starved conditions (Fig. 4C, left), while punctuate LC3-II structures accumulated in the cytoplasm following rapamycin treatment, with intense immunoreactivity for cleaved caspase-3 (Fig. 4C, middle). This change was reversed in ECSCs by co-treatment with rapamycin and 3-MA (Fig. 4C, right). Finally, we conducted flow cytometry assays using annexin-V and PI to determine the proportion of apoptotic cells. As shown in Fig. 4D and E, the proportion of apoptotic ECSCs induced by serum starvation significantly increased by 3.86-fold in the presence of rapamycin. In contrast, the proportion of apoptosis in rapamycin-treated ECSCs then significantly decreased by $\sim 54\%$ after the addition of 3-MA ($P < 0.05$).

**Discussion**

As mTOR functions as a repressor of autophagy, inhibition of mTOR activity is known to efficiently trigger autophagy induction in mammalian cells (Corcelle *et al.*, 2009; Chen and Karantza, 2011). Previous studies have shown that mTOR activity was abnormally increased in endometriotic lesions, compared with normal eutopic endometrium (Yagyu *et al.*, 2006; Honda *et al.*, 2008). In endometriosis, therefore, abnormal mTOR activity may contribute to alteration of autophagy. Consequently, this may affect endometrial cell apoptosis because autophagy induction plays a key role in the regulation of endometrial cell apoptosis during human endometrial cycle (Choi *et al.*, 2012). However, whether autophagy induction is altered by aberrant mTOR activity in endometriosis and is associated with abnormal apoptosis remains unclear. We examined autophagy and mTOR activity in human normal eutopic endometrium and EETs according to the phases of the menstrual cycle. In cycle-dependent endometrial cell autophagy induction in the normal endometrium described by Choi *et al.* (2012), autophagy induction increases as the menstrual cycle progresses, reaching a maximum during the late secretory phase. In this study, we confirmed the cyclic changes in endometrial cell autophagy determined by LC3-II expression and showed that this pattern is inversely correlated with p70S6K phosphorylation, suggesting that a decrease in mTOR activity may be key event in the induction of endometrial cell autophagy during the secretory phase. Furthermore, we also show that the cyclic change of cleaved caspase-3 expression coincides with that of LC3-II. These results indicate that endometrial cell autophagy is induced by inhibition of mTOR activity during the secretory phase and is closely correlated with apoptosis in the normal endometrium. However, previous studies have reported that in patients with endometriosis, apoptotic cyclic variability is not seen and that apoptosis is significantly decreased during the late secretory and menstrual phases in endometrial cells (Johnson *et al.*, 2005;
In endometrial tissues from ovarian endometriotic cysts, our results also demonstrated that no cyclic changes were observed in the expression of LC3-II, phosphorylated p70S6K and cleaved caspase-3 throughout the menstrual cycle, which suggests that, in addition to apoptosis, autophagy and mTOR activity are also constant throughout the menstrual cycle. Therefore, it can be postulated that a constant level of autophagy induction in ovarian endometriotic cysts is mediated by disinhibition of mTOR activity and is related to decreased apoptosis. Indeed, mTOR activity is significantly higher in endometriotic tissues and induction levels of autophagy and apoptosis are lower when comparing the normal endometrium with endometriotic tissues during the late secretory phase. These results suggest that disinhibition of mTOR activity in ovarian endometriotic cysts is involved in the suppression of autophagy induction and the subsequent decrease in apoptosis.

To confirm the in vivo results, we cultured NESCs and ECSCs in vitro with or without the ovarian steroid estrogen and progesterone to mimic physiologic hormonal changes. Previous studies have shown that during the menstrual cycle, endometrial glandular and stromal cell apoptosis are controlled by estrogen and progesterone, which are the two central balancing factors in the human endometrium. Under the influence of estrogen, endometrial cells proliferate as a result of estrogen-induced activation of anti-apoptotic genes and inhibition of pro-apoptotic genes. In contrast, progesterone, the main hormone present during the early-to-mid secretory phase, inhibits estradiol-induced cell proliferation and anti-apoptotic effects in the endometrial epithelium (Rotello et al., 1992; Tabibzadeh, 1995; Selam et al., 2001). In the absence of implantation, the levels of these hormones dramatically decrease during the late secretory phase and are low during menstrual phase, which induces the menstrual cascade, beginning with shrinkage of the entire endometrium, characterized by activation of pro-apoptotic factors and induction of apoptosis (Rudolph-Owen et al., 1998; Song et al., 2002). Similarly, our in vitro experiments showed that the expression of LC3-II in NESCs treated with estrogen alone (proliferative phase) increased with down-regulation of phosphorylated p70S6K after the addition of progesterone (early-to-mid secretory phase) or removal of estrogen and progesterone (late secretory and menstrual phase), followed by an increase in apoptosis. This finding is consistent with our previous report showing that autophagy induction combined with apoptosis increases in endometrial cancer cells following removal of ovarian steroids (Choi et al., 2012). These results suggest that autophagy induction in the normal endometrial cell is mediated by mTOR inhibition during the secretory and menstrual phases and is closely correlated with apoptosis. Furthermore, this in vitro result indicates that autophagy and mTOR activity, as well as apoptosis, seem to be regulated by ovarian steroids in normal endometrial cells. However, previous studies have suggested that endometriotic cells respond abnormally to ovarian steroids, which contributes to dysregulation of apoptosis (Bulun et al., 2006; Reis et al., 2007).
2013). The present study further demonstrated that, in addition to apoptosis, autophagy and mTOR activity did not change in endometriotic cells following treatment with ovarian steroids. Accordingly, endometriotic cell autophagy combined with apoptosis may not be induced due to disinhibition of mTOR activity during the secretory and menstrual phases. This hypothesis is supported by finding that a higher level of mTOR activity was present in ECSCs following withdrawal of estrogen and progesterone compared with NESCs, which inhibited induction levels of autophagy and apoptosis, as shown by the decreased number of autophagosomes and inhibited the expression of LC3-II and cleaved caspase-3.
Taken together, our in vitro results also suggest that endometriotic cell autophagy is suppressed due to disinhibition of mTOR activity during the menstrual cycle, followed by a decrease in apoptosis.

In some cell systems, autophagy induction by mTOR inhibition might be an important cellular mechanism responsible for apoptosis induction (Saiki et al., 2011; Chow et al., 2012; Singh et al., 2012). Our in vivo and in vitro results showed that autophagy induction by mTOR inhibition is closely related to apoptosis induction in both NESCs and ECSCs. Therefore, altered induction of autophagy by aberrant mTOR activity could contribute to abnormal apoptosis in endometriosis. To evaluate this hypothesis, serum-starved ECSCs were treated with rapamycin with and without 3-MA to inhibit mTOR activity or autophagy induction, respectively. Rapamycin treatment induced autophagy and led to the promotion of apoptosis. However, the pro-apoptotic effect of rapamycin treatment was reversed by autophagy inhibition using 3-MA. These results indicate that mTOR inhibition promotes endometriotic cell apoptosis via autophagy induction, suggesting a pro-apoptotic role for autophagy in endometriotic cells. This finding was further supported by immunofluorescence images (Fig. 4C) and flow cytometry assay (Fig. 4D) showing apoptosis induction coincided with rapamycin-induced autophagy. Therefore, it can be postulated that altered induction of autophagy by aberrant mTOR activity is a feasible mechanism that facilitates the decreased apoptosis found in endometriotic tissues.

In conclusion, endometrial cell autophagy is suppressed by disinhibition of mTOR activity during the menstrual cycle in ovarian endometriotic cysts, which results in decreased apoptosis.

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Authors’ roles

J.C. designed the study, interpreted the data and drafted the manuscript. M.J. performed all experiments, interpreted the data and provided critical discussion. E.L. and H.J.K. were involved in sample recruitment. D.C. made substantial contributions to conception and design, interpretation of results and discussion, critical review and editing of the final version of the manuscript.

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Conflict of interest

None declared.

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