European Journal of Obstetrics & Gynecology and Reproductive Biology: X 2 (2019) 100016

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology: X

journal homepage: www.elsevier.com/locate/eurox

Differences in autophagy-associated mRNAs in peritoneal fluid of patients with endometriosis and gynecologic cancers

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ARTICLE INFO

Article history:
Received 13 January 2019
Received in revised form 10 March 2019
Accepted 23 March 2019
Available online 8 April 2019

Keywords:
Autophagy
Endometriosis
Gynecologic cancer

ABSTRACT

Endometriosis and gynecologic cancer show similar patterns of invasion. Little is known about the roles of autophagy in endometriosis and, to date, the expression of autophagy-associated mRNAs has not been compared in patients with endometriosis and gynecologic cancers. This study therefore compared the levels of expression of autophagy-associated mRNAs in patients with endometriosis and gynecologic cancers. The levels of autophagy mRNAs, including those encoding mTOR, P13KC3, Beclin-1, Bcl-2, LC3 II, FLIP, Rubicon, BIRC2 and BIRC5, were measured by real time polymerase chain reaction in peritoneal fluid of 27 patients with benign masses (control group), 42 patients with endometriosis, and 43 patients with gynecologic (ovarian, uterine, and cervical) cancers. Findings in the three groups were compared. Autophagy mRNAs were present in all samples from patients with endometriosis and gynecologic cancers. The levels of PI3K, FLIP, and Rubicon mRNAs were significantly higher in the endometriosis than in the control group ($p < 0.05$ each). Compared with the gynecologic cancer group, the levels of LC3II and FLIP mRNAs were significantly lower, and the levels of Beclin-1 and Rubicon mRNAs significantly higher, in the endometriosis group ($p < 0.05$ each). Levels of PI3K and FLIP mRNA were significantly higher in the endometriosis and gynecologic cancer groups than in the control group ($p < 0.05$ each). PI3K, FLIP, and Rubicon mRNAs are closely associated with the pathogenesis of endometriosis. The similar increases in PI3K and FLIP mRNA expression observed in patients with endometriosis and gynecologic cancer suggest that these conditions have similar autophagic characteristics. The lower levels of Beclin-1 mRNA in the gynecologic cancer than in other two groups suggest that lower Beclin-1 mRNA levels increase the likelihood of developing gynecologic cancer.

Introduction

Endometriosis is a condition in which endometrial tissue (uterine gland and stroma) is present outside the endometrium of the uterus. These ectopic endometrial cells express receptors for various sex hormones, depending on the patient's menstrual cycle. Lesions are characterized by localized bleeding and inflammation, resulting in fibrosis and adhesions and causing pain and infertility. Ectopic endometrial cells can also invade other tissues. Although endometriosis is not malignant, its ability to infiltrate into and invade distant tissues shows the same pattern as metastases of malignant tumors. The etiology and pathogenesis of endometriosis have not yet been clarified, although various hypotheses have been suggested [1,2].

Since autophagy-related genes (Atg) were first detected in yeast, more than 30 Atg genes have been identified [3,4]. Autophagy is a major catabolic process, in which intracellular constituents that are unnecessary or malfunctioning are degraded by lysosomes [5,6]. Autophagy regulates organ development, differentiation and tissue regeneration in various organs by regulating the balance between the synthesis and degradation of cell organs and proteins, i.e., the conversion rate [7]. Autophagy may be induced by deficiencies in intracellular glucose and amino acids, as well as by hypoxia, oxidative stress, and treatment with chemotherapeutic agents. In addition, autophagy has been shown to interact with reactive oxygen species to regulate cellular signaling and protein damage, and to act as a mediator of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease, as well as various

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http://dx.doi.org/10.1016/j.eurox.2019.100016

pathological phenomena such as cancer, diabetes, cardiovascular disease, and inflammatory reactions [8]. Studies on the mechanisms of action and roles of Atgs in various diseases have shown that autophagy plays important roles not only in the degradation of clumps of protein, but in the removal of damaged intracellular organelles and external pathogens. Dysregulation of autophagy is a major cause of diseases such as cancer, metabolic diseases, pathogen infection, otitis media, lung disease, Cohn's disease, degenerative brain disease, and heart disease, and even aging [9–11]. Autophagic processes are elaborately controlled by the proteins they produce, and can be classified into various steps, consisting of initiation, vesicle nucleation, vesicle elongation, fusion, degradation, and termination. The present study analyzed the expression of agonists and antagonists of autophagy. These mTOR is involved in induction and initiation P13KC3 and Beclin-1 are involved in nucleation and BCL-2 acts an antagonist of Beclin-1. LC3 II is involved in autophagosome biogenesis. FLIP is involved in vesicle elongation and Atg antagonistic activity. Rubicon is involved in the inhibition of maturation and BIRC2 & BIRC5 act as antagonists of Rubicon [9–11]. To assess the role of autophagy in the development of endometriosis and gynecologic cancers, this study evaluated the expression of autophagy-associated mRNAs in the peritoneal fluid of patients with endometriosis; benign, non-endometriosis tumors; and gynecologic (ovarian, uterine, and cervical) cancers.

Subjects & methods

Subjects

Intraperitoneal fluid samples were obtained from 112 patients who visited Obstetrics and gynecology department of St. Vincent's Hospital, the Catholic University of Korea and underwent surgery. During laparoscopy, peritoneal fluid was collected aseptically from the Douglas pouch, taking care to avoid bleeding. Patients with inflammatory diseases, hormone producing condition, including pregnancy, and blood contaminated peritoneal fluid or without peritoneal fluid were excluded from the study. The samples were centrifuged at 1800 x g for 10 min; the supernatants were stored at −80 °C in 1.5 ml aliquots; and the cell pellets were stored at −80 °C in 1.5 ml aliquots after adding RNase inhibitor. Findings in the three groups were compared. We excluded patients treated prior to surgery in the gynecologic cancer group in order to eliminate situations that could affect the expression of autophagy by preoperative treatment

The study protocol was approved by the institutional review boards (IRBs) of Vincent's Hospital, The Catholic University of Korea and informed consent was obtained from each patient (VC16TISI0148).

RNA extraction and real-time PCR and CA125 measurment

Total RNA was purified from peritoneal fluid using TRIzol solution following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with 1 μg of total RNA, which was transcribed to cDNA using a reverse transcription system with random hexamers (Promega, Madison, WI, USA) according to the manufacturer's protocol. The primer sequences are shown in Table 1. Real-time PCR was performed on a StepOnePlus real-time PCR system with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR was performed with 1 μl of cDNA in a 20-μl reaction mixture containing 10 μl of Power SYBR Green PCR Master Mix, 2 μl of primers, and 7 μl of PCR-grade water. The reaction conditions were denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The crossing points of the target genes with β-actin were calculated using the formula $2^{-(\text{target gene}-\beta-\text{actin})}$, and relative amounts were quantified.

Table 1
Primers for real-time RT-PCR.

Name	Sequences	annealing temperature	Size (bp)
mTOR	5'-CCTGCCACTGAGAGATGACA-3' 5'-TCCGGCTGCTGTAGCTTATT-3'	60	168
P13KC3	5'-GGAACACCGACCTCACAGTT-3' 5'-CACAGCACCTCCTCTGTGAA-3'	60	128
Beclin-1	5'-AGGTTGAGAAAGGCGAGACA-3' 5'-AATTGTGAGGACACCCAAGC-3'	60	112
Bcl-2	5'-ATGTGTGTGGAGAGCGTCAA-3' 5'-ACAGTTCCACAAAGGCATCC-3'	60	124
LC3 II	5'-AGCAGCATCCAACCAAAATC-3' 5'-CTGTGTCCGTTCACCAACAG-3'	60	187
FLIP	5'-GCAGAGTTTCTGCCAAGGAG-3' 5'-CTCCCAAAGTGCTGGGATTA-3'	60	123
Rubicon	5'-CAGATTCTGCTGCCTCTTCC-3' 5'-AGTGTCTGCCCCTCTGAGAA-3'	60	105
BIRC2	5'-CCAGGTCCCTCGTATCAAAA-3' 5'-GCACGACAAGACTCCTTTC-3'	60	179
BIRC5	5'-GCCCAGTGTTTCTTCTGCTT-3' 5'-TCTCCGCAGTTTCCTCAAAT-3'	60	104
β-Actin	5′-GCGAGAAGATGACCCAGATC-3′ 5′-GGATAGCACAGCCTGGATAG-3′	60	77

Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; mTOR, mechanistic target of rapamycin; P13KC3, class III phosphatidylinositol 3-kinase; Bcl-2, B cell lymphoma 2; LC3 II, light chain 3-II; FLIP, FLICE-inhibitory protein; FLICE, FADD-like IL-1β-converting enzyme; BIRC, baculoviral IAP repeat-containing protein.

We measured the CA 125 test in the patient's serum. We take the patient's blood before the operation and measured the CA125 using an immunoradiometric assay kit (IRMA-Count OM-MA) from DPC (Los Angeles, USA) and the normal cut off was 35 U / ml.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess normality and Levene's test was used to assess the equality of variances between groups. Differences among groups were analyzed by the Kruskal-Wallis test, with the Bonferroni adjusted Mann-Whitney *U* test used for post hoc analysis. We showed mean +/- standard deviation in graph. All statistical analyses were performed using SPSS version 13, with a p-value less than 0.05 considered statistically significant.

Results

Characteristics of patients in the control and experimental groups (Table 2)

Peritoneal fluid was obtained from 27 control patients with benign non-endometriosis tumors (mean age, 42.5 ± 12.6 years), 42 patients with endometriosis (mean age, 36.20 ± 13.5 years), and 43 patients with gynecologic (ovarian, uterine, and cervical) cancers (mean age, 49.2 ± 14.1 years).The concentration of cancer antigen 125 (CA-125) was significantly higher in the cancer group than in the other two groups and significantly higher in the endometriosis than in the control group ($p < 0.05$ each).

Expression of autophagy mRNAs in peritoneal fluid (Fig. 1)

The levels of PI3K, FLIP, and Rubicon mRNAs were significantly higher in the endometriosis than in the control group ($p < 0.05$ each). The levels of LC3II and FLIP mRNA were significantly lower, and the levels of Beclin-1 and Rubicon mRNAs significantly higher in the endometriosis than in the gynecologic cancer group ($p < 0.05$ each). The levels of expression of PI3K and FLIP mRNAs

Table 2
Demographic data.

	control (n = 27)	endometriosis (n = 42)	cancer (n = 43)
Age (years)	42.5 ± 12.6	36.20 ± 13.5	49.2 ± 14.1
Diabetes mellitus	1 (3.7%)	0 (0%)	3 (6.9%)
Hypertension	2 (7.4%)	3 (7.1%)	7 (16.3%)
Menopause	2 (7.4%)	1 (2.3%)	8 (18.6%)
CA-125 (U/ml)*	26.8 ± 18.4	73.9 ± 45.1	205.6 ± 339.1

* $p < 0.05$.

were significantly higher in peritoneal fluid samples from the endometriosis and gynecologic cancer groups than from the control group ($p < 0.05$ each). The expression of mTOR mRNA was similar in all three groups ($p > 0.05$).

Discussion

Endometriosis is a common gynecologic disease affecting 5–15% of women of reproductive age and 20–50% of all infertile women [12,13]. Endometriosis is the most frequent cause of chronic pelvic pain and of dysmenorrhea and infertility in women with chronic pelvic pain [14].

Although the polymeric glycoprotein CA-125 was first identified in the early 1980s as a tumor marker, its role in the body has not yet been accurately determined. The expression of CA-125 is increased in gynecological tumors, such as ovarian and endometrial cancer, as well as in other types of malignant tumors, including pancreatic, lung, breast, colorectal, and gastrointestinal cancer.CA-125 expression is also increased in benign diseases, such as liver cirrhosis (70%), benign ovarian cyst, and pelvic inflammatory disease, as well as during menstruation and the first trimester of pregnancy. Therefore, CA-125 is not appropriate as a diagnostic test for specific diseases. Studies have therefore sought to identify other markers of early endometriosis in serum, peritoneal fluid, and various tissues [15,16]. In agreement with findings showing that CA-125 is expressed in benign tumors, the present study showed that its expression was significantly higher in the endometriosis group than in the control group. Moreover, its expression was significantly higher in the tumor group than in the other two groups, with CA-125 expression correlating with disease severity.

Pathologically, autophagy can have both beneficial and harmful effects. Autophagic processes in neurodegeneration allow for the removal of aggregated proteins before they are toxic and induce the death of neurons that accumulate aggregated proteins. In diabetes, there is a need for maintenance of the structure, mass, and function of β-cells, with an age-associated decline in autophagic activity affecting insulin sensitivity and impairing glucose tolerance. In cardiovascular diseases, increased autophagy can compensate for defects in lysosome function, with defects in the completion of autophagy resulting in the accumulation of autophagosomes that impair cellular function. In immunity, autophagy affects cellular defenses against invasion by bacteria and virus, and may allow replication of nucleic acids by microbial pathogens as well as supplying nutrients for their growth. Autophagy in cancer can act as a tumor suppressor, promoting cell death, or as an oncogene, preventing cell death.

The present study assessed the expression of autophagy-associated mRNAs in groups of patients with benign tumors, endometriosis, and gynecologic malignancies. We were unable to determine whether the expression of these autophagy mRNAs was normal in any of these groups because, for ethical reasons, we could not obtain peritoneal fluid from normal, disease-free individuals. However, autophagy mRNAs were present in all peritoneal fluid samples from the three groups of patients. Autophagy was found to be involved in intraperitoneal tumor immunity. In particular, levels of PI3K, FLIP, and Rubicon mRNAs were significantly higher in the endometriosis than in the control group, suggesting that these autophagy mRNAs were associated with intraperitoneal endometriosis formation. Endometriosis is a chronic inflammatory condition in a pelvic environment that changes to a complex disease characterized by disparate morphological, histological and biochemical properties. Although endometriosis is not a malignant tumor, it is clinically invasive, infiltrating or metastatic, similar to malignant tumors.

The present study found that the expression of PI3K and FLIP mRNA in peritoneal fluid was similar in the gynecologic malignancy and endometriosis groups and was significantly higher in both groups than in the control group. In contrast, the levels of

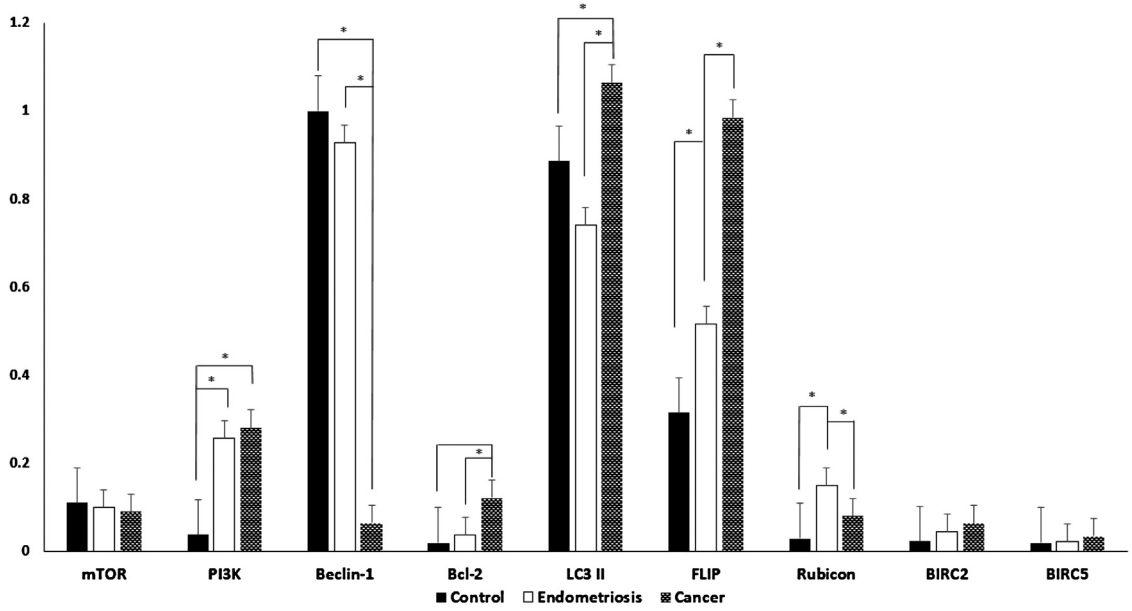

Fig. 1. Comparison of autophagy expression associated mRNAs in control, endometriosis and cancer. Differences among groups were analyzed by the Kruskal-Wallis test, with the Bonferroni adjusted Mann-Whitney *U* test used for post hoc analysis.

LC3II and FLIP mRNAs were significantly lower, and the levels of Beclin-1 and Rubicon mRNAs significantly higher, in the endometriosis than in the gynecologic cancer group. These findings indicate that different autophagy genes are involved in different diseases.

Mechanistically, however, this study could not explain why mTOR mRNA expression were similar in the three groups, and why genes associated with the induction and initiation processes of autophagy did not participate in endometriosis and gynecologic cancer. Moreover, PI3K, which is involved in nucleation in endometriosis and gynecologic cancers, was higher in these two groups than in the control group; and FLIP, which is antagonistic to autophagy-related gene 3 and not an autophagy agonist, was higher in the endometriosis than in the control group. Further studies are needed to determine whether autophagy genes are involved in different stages of endometriosis and gynecologic cancer.

Autophagy plays two roles in tumorigenesis. The first is the induction of apoptosis as a tumor suppressor, and the second is the inhibition of apoptosis during tumorigenesis. In the latter, autophagy can promote cancer cell survival by removing damaged proteins and cellular organelles during conditions of nutrient limitation and hypoxia and following exposure to radioactivity [17–19]. During early tumor stages, inhibition of autophagy can enhance the survival of cancer cells and promotes tumor growth [20,21], whereas, during later stages, autophagy induces cancer cells in the inner part of the tumor, due to the restriction of nutrients, allowing these cells to live in extreme situations; alternatively, these cells may be damaged by therapy to rapidly remove the endoplasmic reticulum. This study found that all autophagy-associated mRNAs, except for Beclin-1 mRNA, were increased in cancer patients. Our finding, that Beclin-1 mRNA was reduced in these patients, was consistent with the results of previous studies [22,23]. The disappearance of Beclin-1, which is related to the formation of autophagosome, has also been observed in breast, uterine, and prostate cancers. Little Beclin-1 protein was detected in the breast cancer cell line MCF7, with MCF7 cells expressing Beclin-1 showing increased autophagic activity and suppression of tumor activity [24]. In addition, beclin-1 +/- mice showed a high rate of tumor formation and beclin-1 - / - mouse stem cells showed a reduced number of autophagy vacuoles [25]. These results suggest that autophagy induces tumor cell death and tumor suppression through growth inhibition.

This study had several limitations. Because we could not obtain peritoneal fluid from healthy subjects due to ethical reasons, we used peritoneal fluid from patients with benign tumors without endometriosis as a control. Second, autophagy mRNAs were assessed in peritoneal fluid rather than lesion tissue. Third, the present study measured mRNAs, not proteins. Fourth, only some autophagy-associated mRNAs were measured in these samples. Fifth, although studies have investigated the association between immune response and the severity of endometriosis, the present study analyzed the association between autophagy mRNAs and the presence or absence of endometriosis.

Conclusions

Autophagy-associated mRNAs were detected in the intraperitoneal fluids of all patients with benign tumors, endometriosis, and gynecologic malignancies, suggesting that these mRNAs may be involved in the pathogenesis of gynecologic diseases.PI3K, FLIP, and Rubicon mRNAs are closely associated with the pathogenesis of endometriosis. The increased expression of PI3K and FLIP mRNAs in the peritoneal fluid of the endometriosis and gynecologic cancer groups suggests that endometriosis and gynecologic cancers have similar autophagic characteristics. The level of Beclin-1 mRNA was lower in the gynecologic malignancy group than in the control and endometriosis groups, suggesting that the decrease in Beclin-1 mRNA expression is associated with the development of gynecologic tumors.

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Financial disclosures

None of the authors reported financial disclosures.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF 2017R1D1A1B3030021).

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