Autophagy is upregulated in ovarian endometriosis: a possible interplay with p53 and heme oxygenase-1

Giulia Allavena, M.S., a,b Patrizia Carrarelli, Ph.D., c Barbara Del Bello, Ph.D., a,b Stefano Luisi, M.D., Ph.D., c Felice Petraglia, M.D., c and Emilia Maellaro, Ph.D. a,b

a Department of Molecular and Developmental Medicine, University of Siena, Siena; b Istituto Toscano Tumori, Florence; and c Obstetrics and Gynecology Unit, Department of Molecular and Developmental Medicine, “S. Maria alle Scotte” Hospital, University of Siena, Siena, Italy

Objective: To evaluate the occurrence of the autophagic process in ovarian endometriomas compared with eutopic endometrium of affected women and with normal endometrium of healthy women.

Design: Biochemical and molecular study in tissue extracts.

Setting: University cellular pathology laboratory and university hospital.

Patient(s): Patients with ovarian endometriosis (n = 13) and healthy women (n = 18).

Intervention(s): Specimens of endometrium were obtained by hysteroscopy from patients with endometriosis and from healthy control subjects; specimens of ovarian endometriomas were collected by laparoscopy. All patients underwent surgery after the end of menstrual bleeding, resulting in most of our patients (approximately 80% in each group) being in the proliferative phase.

Main Outcome Measure(s): Autophagy was evaluated by Western blot analysis of biochemical markers (LC3-II, LC3-II/LC3-I ratio and p62) and by quantitative real-time polymerase chain reaction of autophagy-related genes (ATG14, BECN1, ATG7, and LC3B); apoptosis-related (p53 and Bcl-2) and oxidative stress-related (heme oxygenase-1) proteins were also evaluated by Western blot analysis.

Result(s): All tested biochemical markers and messenger RNA levels of autophagy-related genes showed a significant up-regulation of autophagy in ovarian endometriomas compared with eutopic endometria of affected or healthy women. Moreover, a significant decrease of p53 protein and a significant increase of heme oxygenase-1 protein was also evident in endometriomas.

Conclusion(s): The upregulated autophagic process observed in ovarian endometriomas can be regarded as an integral part of endometriosis pathogenesis, possibly contributing to survival of endometriotic cells in ectopic sites and to lesion maintenance. The decreased susceptibility to apoptosis and the persistent oxidative stress experienced by endometriotic cells could favor autophagy stimulation. (Fertil Steril 2015;103:1244–51. © 2015 by American Society for Reproductive Medicine.)

Key Words: Autophagy, ovarian endometriosis, p53, heme oxygenase-1

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/allavenag-autophagy-ovarian-endometriosis/

Endometriosis is a common gynecologic disease affecting 8%–10% of fertile women and is characterized by pelvic lesions associated with pain and infertility. The pathogenesis of endometriosis is the colonization of the peritoneal cavity by migrating endometrial cells, and the attention is on the mechanisms that promote the establishment and survival of endometrial cells outside of the uterus. The capacity of ectopic endometrial cells to evade the overall control of the apoptotic program is altered in endometriotic lesions and/or in the eutopic endometrium of affected women (1–2), showing an increased expression of proapoptotic factors and a decreased expression of proapoptotic factors compared with the normal endometrium of healthy women. The reduced susceptibility to apoptosis is also accompanied by a few further attributes typical in tumorigenesis and tumor progression (3–4), including abnormal proliferation (5–6), secretion of proteases that contribute to endometrial cell invasiveness through remodelling the extracellular matrix (7, 8), and production of angiogenic factors that promote neovascularisation (9, 10).

In such context, the autophagic response may play a role in endometrial cell survival and lesion maintenance.

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for “QR scanner” in your smartphone’s app store or app marketplace.
Autophagy, commonly referring to macroautophagy, is a physiologically controlled, catabolic process through which cytoplasmic organelles and macromolecules are sequestered in double-membrane autophagosomes and subsequently degraded after lysosomal fusion. The basic components resulting from lysosomal digestion are then reutilized for anabolic processes. It is widely documented that an extensive activation of autophagy is detrimental for cell fate, resulting in autophagic cell death; conversely, a moderate autophagic response acts as a housekeeping, survival mechanism that contributes to maintaining cellular homeostasis in normal conditions or to overcoming stress-induced conditions, including hypoxia, limited nutrients supply, and oxidative stress (11, 12).

The present study aimed to investigate the occurrence of the autophagic process in ovarian endometriotic lesions compared with eutopic endometrium of affected women and with normal endometrium of healthy women; the study also aimed to understand the mechanisms involved in the regulation of the autophagic process.

MATERIALS AND METHODS

Tissue Collection

Samples of endometrium or endometriosis were obtained from nonpregnant women undergoing gynecologic surgery (age range, 21–39 years), with regular menstrual cycle (28–32 days). Samples were divided into [1] the endometriosis group: ovarian endometriotic cysts tissues (endometrioma) (OMA) (n = 13) and their eutopic endometrium (EEOMA) (n = 13), obtained from women undergoing laparoscopic treatment for pain and/or infertility; and [2] the non-endometriosis group: normal endometrial tissues (NE) (n = 18) obtained from healthy women undergoing laparoscopy for tubal sterilization. For each subject, a complete medical history was obtained and physical examination was performed. Patients with hormonal treatment in the past 3 months were excluded from the study. All participants gave written, informed consent before entering this study, which was approved by the local human investigation committee.

The surgical protocol allowed scheduling of all surgical interventions (laparoscopy and hysteroscopy) in the week following the menstrual bleeding, resulting most of our patients (approximately 80%) being in the proliferative phase, as confirmed by ultrasound (13) and by histologic criteria. A specimen of eutopic endometrium was collected by hysteroscopy. In the endometriosis group, the cyst diameter measured by ultrasound ranged from 20 to 80 mm, and all patients showed stage III or IV endometriosis according to the American Society for Reproductive Medicine classification (14). A specimen of endometriotic tissue was collected by laparoscopy; endometrial tissue samples were carefully stripped from the lining inner cyst wall, avoiding contamination with normal ovarian cortex, as confirmed by histologic evaluation.

All specimens were immediately frozen in liquid nitrogen and stored at −80°C until being used for RNA and protein extraction.

RNA Extraction and Complementary DNA Preparation

Frozen tissues samples were cut into small pieces and ground under liquid nitrogen, and total RNA was immediately extracted with the SV Total RNA Isolation System (Promega), according to the manufacturer’s instructions. Ribonucleic acid was quantified by UV absorption, and RNA integrity was preliminarily checked with the FlashGel System (Lonza). To obtain complementary DNA, total RNA (1 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit and random primers (Thermo Fisher Scientific), according to the manufacturer’s instruction.

Gene Expression Analysis by Real-time Polymerase Chain Reaction

The expression level of autophagic genes was measured in triplicate samples by quantitative real-time polymerase chain reaction (PCR) using StepOnePlus and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), with primers listed in Supplemental Table 1 (available online). Primers were designed retrieving RefSeq sequences from the National Center for Biotechnology Information (ATG14: NM_014924.4; Beclin-1: NM_003766.3; ATG7: NM_001136031.2; LC3B: NM_022818.4) and using the Primer-Blast tool to generate suitable primers couple. Sequences were chosen among those spanning between different exons, to limit amplification of genomic DNA. Amplification products were run on a 2% agarose gel to check for predicted fragment sizes. After purification, PCR products were labeled using a dye terminator cycle sequencing kit (Life Technologies), according to the manufacturer’s instructions; DNA sequence was determined using an AB Prism 310 automated genetic analyzer (Life Technologies). For real-time PCR analysis the thermocycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds (for denaturation) and 60°C for 1 minute (for annealing and extension). Melting curve analysis was used to confirm the specificity of the amplified products and the absence of primer–dimer formation. Gene expression values of EEOMA and OMA samples were expressed as fold-change over NE samples, by using the 2−ΔΔCt method (15) with HPRT (hypoxanthine phosphoribosyltransferase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as internal reference genes.

Protein Extraction and Western Blot Analysis

Total proteins from frozen tissue samples were extracted with 0.2% (vol/vol) Triton X-100 buffer, containing 50 mM Tris–HCl (pH 7.5), 10% glycerol (vol/vol), 5 mM magnesium acetate, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM 1,4-dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and Protease Inhibitor Cocktail “Complete” (Roche) as recommended by the supplier. Protein concentration in tissue lysates was determined by using the Bradford (B6916; Sigma) method. Equal amounts (30 μg) of proteins were separated by sodium dodecyl sulfate–polyacrylamide
gel electrophoresis on 4%–20% or 7.5% Mini-Protean TGX Precast gels (Bio-Rad), for 30 minutes at 200 V, and electrophoretically transferred to 0.45- or 0.22-μm nitrocellulose membranes (AppliChem) for 30 minutes at 200 mA. Before adding primary antibodies, quality control and transfer efficiency were assessed by reversible Ponceau S protein staining on membranes. After rinsing, nitrocellulose membranes were blocked for 1 hour at room temperature with 10% skim milk in phosphate-buffered saline (PBS)-Tween 20 0.05% and probed overnight at 4°C with anti-LC3B (microtubule-associated protein 1B-light chain 3) (1:2,000, L7543; Sigma) (LC3B is hereafter referred to as LC3), anti-SQSTM1 (Sequestosome-1) (p62) (1:1,000; Cell Signaling), anti-ATG7 (1:5,000, A2856; Sigma), anti-Beclin-1 (1:5,000, sc-11427; Santa Cruz Biotechnology), anti-p53 (1:1,000, sc-126 [DO-1]; Santa Cruz Biotechnology), anti-Bcl-2 (1:1000, B3170; Sigma), and anti-HO-1 (heme oxygenase-1) (1:1,000, 5061; Cell Signaling Technology). After three washings with PBS-Tween20, membranes were incubated 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit, Sigma, R4880, or goat anti-mouse, Sigma, A5420). Each antibody was diluted in PBS-Tween-1% skim milk. The HRP activity was detected using Clarity Western ECL Substrate (Bio-Rad, 1705061), and membranes were developed on Hyperfilm ECL (Amersham GE Healthcare, 28906835). Each membrane was also probed with anti-β-actin-HRP conjugated antibody (dilution 1:50,000) (Sigma) to normalize protein bands. Densitometric quantification of protein bands was carried out with ImageJ Software 1.46r (Wayne Rasband, National Institutes of Health).

**Statistical Analysis**

Statistical analyses were performed with R software (R version 3.0.2, “Frisbee Sailing”). The Kruskal-Wallis and Mann-Whitney U nonparametric tests were used. A P value of <.05 was considered statistically significant.

**RESULTS**

Biochemical Markers Indicate that Autophagy is Upregulated in OMA

First, the autophagic activity was evaluated in OMA, NE, and EEOMA by measuring the amount of LC3-II and the ratio LC3-II/LC3-I, the most widely used biomarkers of autophagosome formation. With the ongoing autophagic process, LC3B proprotein is first activated to LC3-I by Atg4-mediated proteolysis and then conjugated to the amino group of phosphatidylethanolamine; such conversion to the lipidated form (LC3-II) is crucial for the elongation of autophagosomal membranes and for assisting autophagosome maturation. Thus, the amount of the LC3-II, as well as LC3B conversion (measured as the above ratio), reliably evaluate the formation of autophagosomes. As shown in Figure 1A, B, and D, both LC3B-II and the ratio LC3-II/LC3-I are significantly higher in OMA than in NE and EEOMA.

To further assess the autophagic process, the protein levels of SQSTM1 (p62) were also measured. p62 is a ubiquitin-binding scaffold protein that delivers ubiquitinated protein to the autophagic machinery; because the protein is itself degraded by autophagy, a decline in p62 levels indicates activation of the autophagic flux (16). As shown in Figure 1C and E, the significant decrease of p62 observed in OMA

**FIGURE 1**

Biochemical markers of autophagy indicate that autophagy is upregulated in OMA. Densitometric quantification of LC3-II protein (A), ratio LC3-II/LC3-I (B), and SQSTM1 (p62) protein (C) evaluated by Western blot. Results of protein levels in OMA and eutopic endometria of affected women (EEOMA) are reported as fold-increase over normal endometria from healthy women (NE) (mean value set to 1). (D, E) Representative Western blots.

***P < .001, OMA vs. NE or EEOMA.

compared with NE and EEOMA supports an increased autophagic process in OMA.

**Autophagy-related Genes are Upregulated in OMA**

At present, more than 40 proteins have been identified in macroautophagy (commonly referred to as autophagy). These proteins can be divided in several groups, according to the pathway stage at which they act: induction, nucleation and expansion, fusion, and degradation or efflux (17). Here we evaluated the messenger RNA (mRNA) expression levels of four autophagy-related genes (ATGs), involved in critical steps of the autophagic pathway. ATG14 and BECN1, coding for two major components (Atg14 and Beclin-1) of the Vps34 complex, necessary for autophagic membrane nucleation and lately for autophagosome-lysosome fusion, were significantly higher in OMA than in NE and EEOMA (Fig. 2A and B). This transcriptional induction of autophagic genes in OMA is further confirmed by the significant up-regulation of ATG7 and LC3 genes (Fig. 2C and D), both necessary for membrane expansion and closure. In particular, ATG7 codes for an activating enzyme involved in the formation of Atg5-Atg12 complex and in the conjugation of LC3 to phosphatidylethanolamine. At variance with their mRNA levels, Beclin-1 and Atg7 protein levels are slightly decreased in OMA compared with NE and/or EEOMA (Fig. 3).

**p53 Protein is Dramatically Down-regulated in OMA**

To investigate whether other biological events involved in the pathophysiology of endometriosis were correlated to the enhanced autophagic process observed in OMA, we measured the expression levels of two key molecules entailed in the apoptotic process: p53, a tumor suppressor protein that negatively regulates cell proliferation and induces apoptotic cell death (18), and Bcl-2, an oncoprotein that inhibits apoptotic cell death (19). Despite a notable variability of p53 protein levels in the EEOMA group, p53 is dramatically and significantly down-regulated in OMA compared with both NE and EEOMA (Fig. 4A and D); Bcl-2 protein is also slightly but significantly down-regulated in OMA compared with NE (Fig. 4B and E).

**HO-1 is Induced in OMA**

Because the molecular milieu inside the ovarian endometriotic cyst is particularly rich with free, redox active iron, it is assumed that endometriotic cells experience a persistent condition of oxidative stress. In this perspective, we measured the expression level of HO-1, the rate-limiting enzyme in heme degradation, highly inducible by oxidative stress and inflammation (20). HO-1, whose expression is almost undetectable or very low in NE and EEOMA, is notably up-regulated in OMA (Fig. 4C and F).

---

**FIGURE 2**

Autophagy-related genes are upregulated in OMA. Messenger RNA levels of ATG14 (A), BECN1 (B), ATG7 (C), and LC3B (LC3) (D). Gene expression values of EEOMA and OMA samples, measured by quantitative real-time PCR, are expressed as fold-change over NE samples. *P<.05 and ***P<.001, OMA vs. NE or EEOMA.

DISCUSSION

Autophagy is a complex catabolic process through which macromolecules, cellular organelles, and intracellular pathogens are sequestered in autophagosomes and subsequently digested after fusion of autophagosomes with lysosomes. This process is generally regarded as a survival mechanism contributing to maintenance of cellular homeostasis under both normal and stress-induced conditions (11, 12, 21). Compared with normal endometria of NE and EEOMA specimens, in ovarian endometriomas (OMA) we observe a remarkable activation of the autophagic process, evaluated by means of functional parameters (i.e., the significant
increase of lipidated LC3-II protein levels and LC3-II/LC3-I ratio and the significant decrease of the autophagic substrate SQSTM1 [p62] protein. Moreover, a significant transcriptional induction of LC3B, ATG14, BECN1, and ATG7 genes, coding for proteins involved in different steps of the autophagic pathway, is also evident. At variance with their mRNA levels, Beclin-1 and Atg7 protein levels are slightly lower in OMA than in NE or EEOMA, suggesting that these proteins, being recruited in an increased autophagic flux, can undergo a more rapid turnover, afforded by the autophagic process itself or by different proteolytic mechanisms. In a pathophysiological perspective, such autophagy activation occurring in OMA can be regarded as a further adaptive mechanism that contributes, in addition to the reduced susceptibility to apoptotic cell death [1], to survival of endometrial cells in ectopic sites and to lesion maintenance.

Rather than being two independent events, autophagy and apoptosis are two cross-talking mechanisms, and in the majority of experimental settings they prove to be mutually inhibitory [22]. Thus, in endometriotic cells, the well-documented inefficiency to execute the apoptotic death program can lead to an increased autophagic process, as observed by us; on the other hand, the enhanced autophagic response could be in charge of suppressing the apoptotic process [22]. Such pathogenetic hierarchy is difficult to be ascertained in ex vivo investigations; however, the dramatic loss of the master inducer of apoptosis, p53, observed by us in OMA, besides suggesting an impaired apoptosis, can also account for the stimulation of autophagy. In fact, it has been demonstrated that low cytosolic levels of p53, as obtained by pharmacologic inhibition or genetic depletion/deletion, can trigger autophagy [23], likely through derepression of the autophagy-initiating ULK1 complex [24]. To explain the p53 down-regulation, two major autophagy-independent mechanisms can be evoked, both related to Akt pathway, the latter proved to be overactivated in endometriotic tissues and cells from ovarian endometriomas [25, 26]: the first mechanism is the transcriptional down-regulation of p53, through Akt-mediated phosphorylation and inhibition of the transcription factor PHF20/TZIP [27]; the second is the posttranscriptional degradation of p53, through Akt-mediated activation of Mdm2 [28].

In the cross-talk between apoptosis and autophagy, the antiapoptotic protein Bcl-2 can also play a role. The decreased levels of Bcl-2 we find in OMA would paradoxically suggest that endometriotic cells are more prone to undergo apoptosis. However, it is well known that the expression levels of other antiapoptotic proteins of the same family, as well as the heterodimerization of Bcl-2 with the proapoptotic protein Bax, are also crucial to determine susceptibility to apoptosis [29]. Rather, because Bcl-2 binds Beclin-1 to suppress autophagy [30, 31], it is conceivable that lowered levels of Bcl-2 disengage Beclin-1 to sustain the autophagic process.

The up-regulation of the Akt pathway demonstrated in endometriotic tissues has been suggested to promote the survival of endometriotic cells [32]. The Akt pathway is also known to activate mammalian target of rapamycin (mTOR), which in turn inhibits the autophagy initiating ULK1 kinase complex [33]. Thus, Akt up-regulation, along with mTOR up-regulation also found in endometriosis [34, 35], would be expected to impair the autophagic response. Consistently, in a recently published article [36] a correlation between p70S6K phosphorylation (signature of mTOR activation) and lower protein levels of LC3-II was reported in ovarian endometriotic cysts. Differently, in the present study we extensively demonstrate, by measuring gene expression and protein level changes of several autophagic effectors and markers, that a significantly increased autophagic process occurs in ovarian endometriomas compared with eutopic endometria. Our findings are compatible with a concomitant Akt/mTOR up-regulation, because autophagy can be also induced by mTOR-independent mechanisms, as demonstrated for several compounds, including Ca++ channel blockers, adenylyl cyclase inhibitors, calpain inhibitors, and trehalose [21, 37].

A good candidate responsible for autophagy induction in endometriotic cells is oxidative stress. Because of the cyclic bleeding in endometriotic tissues, the hemoglobin released from hemolysis leads to accumulation of high levels of heme. Heme undergoes the heme oxygenase-catalyzed breakdown to biliverdin (then converted to bilirubin), carbon monoxide, and iron [38]. In fact, the fluid of endometriotic ovarian cysts contains non–protein bound (catalytic) iron, reactive oxygen species (ROS), and lipid peroxides in concentrations from one to two orders higher than those found in nonendometriotic cysts [39, 40]; consequently, they experience a strong and persistent oxidative stress that can induce autophagy to eliminate ROS-damaged organelles and proteins. Heme oxygenase is a microsomal enzyme present in three isoforms (HO-1, -2, and -3), among which HO-1 (also known as heat shock protein 32 [HSP32]) is inducible by several oxidant and nonoxidant stressors [20]. Although the above end-products are cytotoxic at high concentrations, induction of HO-1 proves to be an adaptive defence mechanism, able to protect cells in a large number of unrelated pathologies [20]. In addition to its role as a negative modulator of inflammation and apoptosis, HO-1 can also exert such cytoprotection by stimulating autophagy [41–44]; the autophagic response has been ascribed, among other involved mechanisms, to the mitochondrial ROS generation promoted by the HO-catalyzed production of carbon monoxide [45]. In normal human endometrium, the major isoforms HO-1 and HO-2 are expressed [46, 47]. Although in adenomyosis the expression level of HO-1 and HO-2 was found to be lower than those in the eutopic endometrium [48], high levels of transcript and intense immunohistochemical staining of both isoforms have been detected in peritoneal endometriotic biopsies (particularly in red lesions), along with high concentrations of hemoglobin in peritoneal fluids [49]. In the present study we find a much higher expression of HO-1 protein in ovarian OMA than in NE or EEOMA. This finding suggests that a condition of persistent but tolerated oxidative stress, per se [50–52] or through a positive feedback with HO-1, can stimulate the autophagic process.

We performed our investigations in specimens (mostly resulting in the proliferative phase of the menstrual cycle in each group) that we analyzed collectively. The lack of a subanalysis according to the menstrual cycle, and possible differences in tissue composition between eutopic and ectopic
endometrium (e.g., percentage of immune cells, and glandular/stromal cells ratio) are limitations of our study, deserving further insights.

In conclusion, we show that in ovarian endometriomas the autophagic process is up-regulated, as evaluated by several biochemical and molecular indicators. Such enhanced defence mechanism, possibly contributing to survival of endometriotic cells in ectopic sites and to lesion maintenance, can be regarded as an integral part of endometriosis pathogenesis. Two mechanisms can be responsible for autophagy stimulation: the decreased susceptibility to apoptosis, which in turn is controlled by autophagy; and the persistent oxidative stress, by itself and/or its down-stream response. Future investigations on cell lines derived from endometriotic lesions will provide a reliable experimental system to dissect this pathophysiologic network.

REFERENCES


### SUPPLEMENTAL TABLE 1

**Primers used for gene expression analysis by real-time PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>For 5'-</th>
<th>Rev 5'-</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG14</td>
<td>-CTGC(\text{GCCAAATGCGTTCAG-3'})</td>
<td>-GTCGATAAACCTCTCCCGGTTC-3'</td>
</tr>
<tr>
<td>BECN1</td>
<td>-TAGACC(\text{GGACTTTGAGTGACG-3'})</td>
<td>-TAGACCC(\text{CTCCCCCTCACAG-3'})</td>
</tr>
<tr>
<td>ATG7</td>
<td>-CGGC(\text{GCAAGAAATAATG-3'})</td>
<td>-CCCAAC(\text{ATCCCAAGCACACTAC-3'})</td>
</tr>
<tr>
<td>LC3B</td>
<td>-CGGC(\text{ACCTCCGAACAAAGAG-3'})</td>
<td>-A(\text{AGCTGCTTCTCACCTTTG-3'})</td>
</tr>
<tr>
<td>HPRT</td>
<td>-CGTG(\text{ATTAGTGATGATGAACCAG-3'})</td>
<td>-CGAGCAAGACGTTCAGTCTTC(\text{GTG-3'})</td>
</tr>
<tr>
<td>GAPDH</td>
<td>-GAAGG(\text{GTGAAGGTCGAGTTC-3'})</td>
<td>-GAAGAT(\text{GTGGATGGAATTC-3'})</td>
</tr>
</tbody>
</table>