Proteolytic tailoring of the heat shock protein 70 and its implications in the pathogenesis of endometriosis

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Objective: To investigate the mechanism underlying the appearance of a 20-kd HSP70 fragment and its consequences in the ectopic endometrium of endometriosis patients.
Design: Experimental study.
Setting: Research institute and obstetrics and gynecology clinic.
Patient(s): Participants with (n = 18) and without (n = 20) endometriosis.
Intervention(s): None.
Main Outcome Measure(s): Reverse-transcription polymerase chain reaction, protease assays, and in silico tools were used to investigate the origin of the 20-kd HSP70 fragment. Immunocolocalization studies were carried out to determine whether subtilisin/kexin isozyme 1 (SKI-1) and HSP70 are colocalized. Expression and localization of surrogate markers of inflammation, such as nuclear factor NF-κB and interleukin IL-6 were examined by immunoblotting and in situ studies.
Result(s): HSP70 is posttranslationally processed into a 20-kd fragment by SKI-1, a protease of the subtilisin family, in ectopic endometrium (ECE). Immunocolocalization studies revealed spatial proximity of SKI-1 and HSP70 in ECE. Furthermore, ECE demonstrated nuclear localization of the transcription factor, NF-κB and high expression of its target protein, IL-6.
Conclusion(s): This study hints at the possible mechanisms underlying the trimming of HSP70 in ECE and also at the role of proteases in the pathogenesis of endometriosis. The possible repercussions of HSP70 fragmentation include dysregulation of key regulatory proteins, resulting in the escalation of inflammatory events in endometriotic lesions. (Fertil Steril® 2011;95:1560–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, ectopic, HSP70, NF-κB, IL-6, SKI-1, proteases

Endometriosis is defined by the growth of endometrial-like glands or stroma at extrauterine sites. It clinically manifests as dysmenorrhea, chronic pelvic pain, and infertility in 10%–15% of women of reproductive age (1). The histogenesis of endometriosis is attributed to the implantation of menstrual endometrial tissue into the abdominal cavity through reflux via the fallopian tubes (2).

Our interest has been to gain an insight into the events underlying the molecular transformation of eutopic endometrium into an endometriotic lesion in the backdrop of the same genotype. Previously, using the two-dimensional proteomics approach, we have identified proteins that are overexpressed in ectopic (extrauterine) endometrium (ECE) compared with their eutopic (intrauterine) counterparts (EUE) in endometriosis patients as well as compared with endometrium of regularly cycling women (3). One of these proteins was ~20 kd and was identified as being a fragment of the heat shock protein 70 protein 8 (HSPA8) (Supplementary Fig. 1, available online).

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HSPs are highly conserved proteins, classified according to their molecular weight. Of these, the HSP70 family is known to possess 12 members (4), one of these is HSPA8, or HSC70. HSP70 proteins function as cell chaperones where they assist newly synthesized proteins in processes such as folding and translocation as well as maintain general quality control (5).

Fragmentation of HSP70, especially in the EUE, was intriguing. Fragments/variants of HSP70 have been reported earlier. Freeman et al. demonstrated proteolytic sensitivity of mutant human HSP70 toward trypsin, resulting in 45-kd, 25-kd, and 18-kd fragments (6). A 54-kd splice variant of human HSC70 (HSC54), was first identified in the U937 promonocytic cell line and in certain somatic tissues (7). Recently, a 37-kd fragment of HSC54 was found differentially expressed between normal pancreatic, pancreatitis, and pancreatic adenocarcinoma tissue (8). Neuroendocrine tumours also express a truncated variant of HSPA8, but of 40 kd (9). These studies indicate that the HSP70 family not only encompasses the conventional ~70-kd members but also variants of smaller size originating owing to either transcriptional or proteomic amendments to the parent molecule.

The 20-kd fragment of HSP70 is a new addition to the HSP70 family, and its ectopic tissue specific expression prompted us to investigate the origin and probable outcome of HSP70 cleavage.

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Toward this end, we investigated whether the 20-kd fragment originates from an alternate transcript or owing to posttranslational events, such as proteolysis. There have been studies demonstrating association between HSP70 and transcriptional factors, such as nuclear factor NF-κB (10–12). Therefore, NF-κB–regulated events were expected to be impaired in endometriotic tissue in the wake of HSP70 fragmentation. The expressions of NF-κB and its downstream target, interleukin (IL) 6, were also investigated. The observations hint toward a possible link between HSP70 fragmentation and inflammation, a key feature of endometriotic lesions.

MATERIALS AND METHODS

Participants

The study was approved by the Ethics Committees of the National Institute for Research in Reproductive Health and the Sanjeevani Diagnostic Center, Mumbai. Informed written consent was obtained from each of the participants (aged 22-35 years). Women with a history of pelvic inflammatory disease, malignancy, infections, bleeding disorders, or intake of GnRH agonists or steroids ≤3 months before surgery were excluded from the study.

Paired EUE and ECE tissue biopsies (n = 18) were obtained from women undergoing diagnostic or therapeutic laparoscopy who presented with mild to severe endometriosis according to the revised American Fertility Society guidelines (13). Control samples (CE) were also collected from regularly cycling women (n = 20) with no visual evidence of endometriosis on laparoscopy. Endometrial samples were dated (14), and both phases of the menstrual cycle were adequately represented in each experiment so as to avoid phase-dependent changes from influencing the results.

Antibodies

HSP70 mouse monoclonal antibody (mMAb; MA3-008; Affinity Bioreagents) was used at 1:4,000 dilution in immunoblots and 1:500 in tissue sections (TS). Rabbit polyclonal subtilisin/kexin isozyme 1 (SKI-1; sc-20757) and IL-6 (sc-7920) were used at 1:50 dilution in immunoblots and TS (Santa Cruz Biotechnology). NF-κB p65 mMAb, (BD Transduction Laboratories) was used at 1:100 dilution in TS and immunoblots. GAPDH mMAb (Calbiochem) was used at 1:5,000 dilution. For immunoblotting, both secondary antibodies, swine antirabbit–horseradish peroxidase (HRP) and rabbit antimouse-HRP, were used at 1:3,000 dilution (DakoCytomation).

One-Dimensional Immunoblot

Protein extracts (30 μg each) of EUE, ECE, and CE were processed for immunoblotting as previously described (3). The blots were stripped using the Restore Western Blot Stripping Buffer (Pierce) and reprobed for GAPDH.

Reverse-Transcription Polymerase Chain Reaction

RNA samples extracted from three paired EUE and ECE, and four CE tissues were reverse transcribed into cDNA using the ImProm-II Reverse Transcription System (Promega) and amplified for HSPA8 by polymerase chain reaction (PCR) using different primer sets.

Zymography and Quantitative Protease Assay

Control (n = 6) and paired EUE and ECE (n = 5 each) collected in 0.1% Triton X-100 were separated on 0.5% gelatin-impregnated 12% polyacrylamide gels and processed for zymographic analysis as previously described (15).

The QuantiCleave Protease Assay Kit (Pierce) was used as per the manufacturer's instructions. Statistical differences in total protease activity between ECE, EUE, and CE were calculated by unpaired *t* test with Welch's correction.

Protease Identification

The protein sequence of heat shock 70-kd protein 8 (UniProtKB accession number: Q53HF2_Human) was submitted to an online protein characterization tool called Eukaryotic Linear Motif (ELM). ELM is a resource within the Expert Protein Analysis System (ExPASy) proteomics server for predicting functional sites/motifs in eukaryotic proteins. The ELM search after globular domain-filtering, structural-filtering, and context-filtering using the default parameters, in addition to specifying the species as *Homo sapiens*, listed proteases along with their cleavage sites. Furthermore, computational analysis of the theoretic isoelectric point (pI) and molecular weight (Mw) for each of the possible fragments generated with these proteases was carried out by using the "Compute pI/Mw" tool within the ExPASy server.

In Vitro Analysis to Determine Subtilisin Activity on HSP70

Subtilisin was partially purified from batch cultures of *Bacillus subtilis NCIM 2713* (16) and its identity confirmed by tandem mass spectrometry (MS/MS), and activity assayed using the QuantiCleave Protease Assay Kit (Pierce).

To determine whether subtilisin can cleave HSP70 protein, 1:1 ratio of the partially purified subtilisin and human recombinant HSP70 protein (hrHSP70; H7283; Sigma-Aldrich) were allowed to react at 37°C for 15 minutes and probed with the HSP70 antibody.

In Situ Localization of Proteins in Endometrial Tissues

CE and paired EUE and ECE tissues (n = 6 for each category, same as for immunoblotting) were collected in chilled 0.01 mol/L phosphate-buffered saline solution (1×PBS) containing the Complete protease inhibitor cocktail (Roche) and processed as previously described (3). SKI-1 was immunolocalized by using the Alexa Fluor594 goat antirabbit SFX kit (Molecular Probes, Invitrogen) and HSP70 and NF-κB p65 with the Alexa Fluor488 goat antimouse SFX kit (Molecular Probes, Invitrogen). For NF-κB p65, antigen retrieval was carried out as previously described (17). Secondary antibodies were added along with 4,6-diamidino-2-phenylindole for 1 hour at room temperature. Slides were mounted in ProLong Gold antifade reagent (Molecular Probes, Invitrogen). Dual-stained images were captured by sequential scanning using Carl Zeiss LSM510 Meta Confocal system and colocalization quantified using Zeiss LSM software version 4.0. IL-6 was immunolocalized using the Vectastain Elite ABC-peroxidase kit (Vector Laboratories) as previously described (3).

RESULTS

HSP70 Fragmentation in Ectopic Endometrium

Immunoblotting with HSP70 antibody revealed presence of a 20-kd doublet in eight out of ten ectopic tissue lysates. In four out of these eight samples, an additional 35-kd band was also present (Fig. 1A). In some ectopic tissues, the 70-kd band was undetectable, though fragments of 20 and 35 kd were seen (Fig. 1A, lanes EC2 and EC3). In contrast, none of the corresponding eutopic or control endometrial lysates showed any reactivity other than at the expected 70-kd position (Fig. 1A). Thus, a 20-kd HSP70 variant was detected in ectopic endometrium alone.

HSP70 Transcript Size in Ectopic Endometrium

To determine whether HSP70 protein variants originate from transcriptional or posttranscriptional modifications in ectopic tissues, reverse-transcription (RT) PCR analysis of CE, EUE, and ECE tissues was carried out using four different primer pairs spanning the entire HSPA8 region (Fig. 1B; Supplemental Fig. 2, available online at www.fertstert.org). Amplicons of sizes expected to originate from a complete transcript were obtained. Primer pair F1+R3 resulted in a ~650-bp PCR product, F2+R2 in a ~740-bp PCR product, F3+R1 in a ~950-bp PCR product, and F4+R1 in a ~650-bp PCR product in CE, EUE, and ECE (Fig. 1C). Smaller-sized transcripts that could result in 35-kd or 20-kd truncated proteins were not observed.