Role of cathepsin E in decidual macrophage of patients with recurrent miscarriage

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ABSTRACT: In a previous study, we reported that the cathepsin-cystatin system caused endometrial dysfunction in early pregnancy. Here, we investigated the existence and contribution of cathepsin E in early pregnancy in patients with recurrent miscarriage (RM). The effect of cathepsin deficiency on fertility and female reproductive organs were also analyzed in CatE−/− mice. Human studies were conducted in a hospital setting, with informed consent. Cervical mucus was collected from RM patients in early pregnancy (4–6 gestational weeks, n = 21), and the pregnancy outcome was compared prospectively. The cathepsin E expression in decidua of RM patients (n = 49) and normal pregnant women undergoing elective surgical abortion (n = 24) was measured using SDS–PAGE, and western blot analysis. Decidual macrophages were isolated from RM patients (n = 6) and stimulated by lipopolysaccharide (LPS) and interferon gamma (IFN-γ). Results from the mouse model showed that CatE−/− mice were fertile, but the litter number was significantly smaller. The uterus of CatE−/− mice showed granulation tissue. In human samples, protease activity of cathepsin E measured with Fluorochrome-Quenching Substrate (KYS-1) in cervical mucus of patients who developed miscarriage was markedly decreased compared with patients without RM. The expression of cathepsin E in decidua, semi-quantified by SDS–PAGE, western blot analysis was significantly lower in RM patients compared with patients without RM. By double staining immunofluorescence, the staining of cathepsin E was observed in CD14 or CD68 positive cells in all deciduas. Upon stimulation with LPS and IFN-γ, the expression of cathepsin E in cell lysate of decidual macrophages was markedly reduced in RM patients compared with controls. The results suggested that decreased activity of cathepsin E produced by decidual macrophages might be responsible for the induction of miscarriages in some RM patients.

Key words: cathepsin E / decidua / macrophage / proteolysis / recurrent miscarriage

Introduction

Miscarriage, or pregnancy loss, is one of the most common reproductive problems in humans. Putative explanations of recurrent miscarriages (RM) are chromosomal abnormalities in the fetus, as well as antiphospholipid (aPL) antibodies and uterine anomalies (Farquharson et al., 1984; Raga et al., 1997; Sugiuara-Ogasawara et al., 2004), although it has been reported that as much as 24.5% of RM remain unexplained (Sugiura-Ogasawara et al., 2012). Immunological factors may also contribute to human reproductive failure via infertility or spontaneous miscarriage, although, at present, efficacious immunotherapy protocols for general application have not been established (Lambropoulou et al., 2006). A recent report suggested that decidual macrophages play an important role in some women with recurrent miscarriage (Jin et al., 2009). We have reported that serum M-CSF may play an important role in the maintenance of pregnancy (Katano et al., 1997), and also reported that cervical IL-6 and IL-8, which are inflammatory cytokines produced by macrophages, might have predictive value for cases of recurrent miscarriage (Hattori et al., 2007). In patients with antiphospholipid syndrome, interaction of β2-glycoprotein I with lipopolysaccharide (LPS) leads to Toll-like receptor 4 (TLR4)-dependent activation of macrophages (Mulla et al., 2009). A recent report demonstrated that aPL induce a placentinal inflammatory response in first trimester via the TLR-4/MyD88 pathway (Laplante et al., 2011).
Cathepsin E is an endolysosomal aspartic proteinase of the pepsin superfamily, that is expressed predominantly in cells of the immune system and is highly secreted as the catalytic enzyme by activated phagocytes (Sakai et al., 1989; Sastradipura et al., 1998; Nishioku et al., 2002). Cathepsin E plays an important role in cancer immunology, preventing tumor growth and metastasis in vivo through multiple mechanisms, such as induction of apoptosis, inhibition of angiogenesis and enhanced immune responses (Shin et al., 2007). Recently, cathepsin E has been reported as a useful early diagnostic target and an effective drug activator for pancreatic ductal adenocarcinoma (Cruz-Monserrate et al., 2012; Kelhier et al., 2013). Moreover, cathepsin E plays a substantial role in host defense against tumor cells through TRAIL-dependent apoptosis and/or tumor-associated macrophage-mediated cytotoxicity since macrophage infiltration is profound in cathepsin E transgenic mice administered human tumor xenografts (Kawakubo et al., 2007). In prostate cancer, cathepsin E may have therapeutic potential for use in conjunction with chemotherapy as it has been suggested that it can overcome tolerance to chemotherapy in the cancer cells (Yasukochi et al., 2010).

On the other hand, cathepsin E plays a substantial role in immune responses against micro-organisms. For example, previous studies have shown that cathepsin E deficient (CatE−/−) mice display aberrant immune reactions such as atopic dermatitis and higher susceptibility to bacterial infection (Tsukuba et al., 2003). The precise mechanisms underlying abnormal immune responses induced by cathepsin E deficiency are not fully understood, but there are many reports suggesting that cathepsin E regulates the nature and function of dendritic cells and macrophages (Kakehashi et al., 2007; Yanagawa et al., 2007; Li et al., 2008; Tsukuba et al., 2009). Cathepsin E increases the cell surface expression of TLR2 and TLR4 required for innate immune responses (Tsukuba et al., 2006), and it also has a nonredundant role in the class II MHC antigen processing pathway within dendritic cells (Chain et al., 2005).

We have reported that the regulation of the cysteine cathepsin-cystatin system may play an important role in patients with RM. The concentration of cathepsin B and H in patients’ decidua was significantly higher, and the serum level of cystatin C was significantly lower than in control individuals (Nakanishi et al., 2005). Moreover, it was reported that the expression rates of cathepsin B and L differ in decidua of early pregnancy between spontaneous abortion and artificial abortion, suggesting that the cathepsins may play important roles in the process of implantation (Wang et al., 2005).

However, the role of cathepsin E in miscarriage in human reproduction has not been investigated, and in this study, we explored the existence and contribution of cathepsin E in early pregnancy of patients with RM.

### Materials and Methods

#### Analysis of cathepsin E knockout mice

Wild-type and Cathepsin-deficient (CatE−/−) mice on the C57BL/6 genetic background were used as described previously (Tsukuba et al., 2003). The use and care of animals were reviewed and approved by the Animal Research Committee of the Graduate School of Pharmaceutical Science, Kyushu University, Japan. All animals were maintained according to the guidelines of the Japanese Pharmaceutical Society in a specific pathogen-free facility at the Kyushu University Station for Collaborative Research. To assess the fertility of CatE−/− mice, the number of littersmates was counted. Laparotomy was performed at age 8 or 52 weeks.

### Patients

All patients were managed at Nagoya City University Hospital from April 2008 to March 2010. The patients had a history of two or more spontaneous miscarriages. Hysterosalpingography, chromosome analysis for both partners, immunological tests for parameters such as natural killer cell activity (Kato et al., 2013), determination of antiphospholipid antibodies (aPLs) including lupus anticoagulant by activated partial thromboplastin time, diluted Russel viper venom time and β2 glycoprotein I-dependent anticardiolipin antibody method (Ogasawara et al., 1996), blood tests of free thyroxine (T4), thyroid stimulating hormone (TSH), blood glucose and prolactin (PRL) were performed on all patients before subsequent pregnancy. Informed consent, approved by the Institutional Review Board, was obtained from all subjects before collection of any materials.

#### Protease activity assay of cathepsin E in cervical mucus

The protease activity of cathepsin E in cervical mucus (CM) was analyzed from RM patients with early pregnancy (4–6 gestational weeks, n = 21), and the pregnancy outcome was compared prospectively. All patients were followed up with no medications. CM was collected from the endocervical canal using an absorbent cotton swab (Osaki Medical Corporation, Nagoya, Japan) placed into the cervical os for a minimum of 1 min after visualization of the cervix. CM was diluted in phosphate-buffered saline (PBS) and sonicated using a sonifier (Branson Ultrasonic Corporation, Danbury, CT, USA) at power M, level 4 for 5 min at 4°C until solubilized (Menge and Naz, 1993). After centrifugation at 12,000 × g for 30 min at 4°C, the supernatant fractions were aliquoted and stored at −80°C until used in assays as CM samples. Samples were analyzed within a year, avoiding repeated thaw-freeze cycle. The protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) by reading the absorbance at 562 nm using a plate reader (SpectraMax 340 with SoftMax Pro ver. 5.2, Molecular Devices, Inc., CA, USA). Cathepsin E activity in the CM was determined using cathepsin E-specific fluorescence-Quenching Substrate KYS-1: MOCAC-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH2 (Peptide Institute, Inc., Osaka, Japan) as a substrate according to the method described previously (Yasuda et al., 2005) with fluorescence micro plate reader (SpectraMax Gemini EM with SoftMax Pro ver. 5.4.5, Molecular Devices).

#### Measurements of cathepsin E in decidual tissue

Decidual samples were surgically collected from 49 patients with RM, under the diagnosis of missed abortion. Control samples were obtained from 24 women with normal pregnancies, undergoing elective surgical abortions. The patients and controls for this analysis are completely independent group from the cohort of CM analysis. We performed dilation and curettage in both groups and separated decidua from tissue macroscopically with informed consent. The average age in the patient group was 33.0 ± 2.0 years (range 25–44) and 31.0 ± 2.4 years (range 25–38) in the control group. Mean gestational age in the patient group was 8.0 ± 1.0 weeks (range 5–10 weeks) and 7.0 ± 1.4 weeks (range 6–11 weeks) in the control group. The decidual tissues were separated macroscopically in wash buffer [20 mM Tris–HCl, 5 mM ethylenediaminetetraacetic acid (EDTA)-2Na, 1 mM ethyleneglycoltetracetic acid (EGTA), 10 mM 2-mercaptoethanol, 10 μM p-4-amidinophenylmethane sulfonfluoride hydrochloride (APMSF), 150 mM NaCl, 0.25% protease inhibitor cocktail (Sigma, St. Louis, MO, USA), pH 7.5] and stored at −20°C until use. For analysis, the samples were sonicated in lysing buffer.
using a sonifier (Branson Ultrasonic Corporation) at a duty cycle of 50% and output control at 8 for 1 min at 4°C. Then, the samples were centrifuged at 8000 × g for 30 min at 4°C, the supernatant fraction was collected and the protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific, Inc.) by reading the absorbance at 562 nm using plate reader (SpectraMax 340 with SoftMax Pro ver. 5.2, Molecular Devices).

Decidual macrophage isolation and cultures

We collected the samples surgically from six patients with RM managed in Nagoya City University Hospital, under the diagnosis of missed abortion. We performed dilation and curettage and separated decidua from tissue macroscopically. The tissue was washed in saline, cut into small pieces and suspended in 0.05% Trypsin-EDTA (Life Technologies Corporation, Carlsbad, CA, USA) at room temperature for 5 min. RPMI-1640 culture medium (Life Technologies Corporation) containing 1 mg/ml collagenase (Life Technologies Corporation) which has low protease activity, DNase I (100 μg/ml) (Roche Diagnostics, Basel, Switzerland), 1% penicillin–streptomycin–glutamine (Life Technologies Corporation) and 10% fetal bovine serum (Life Technologies Corporation) at 37°C for 60 min. The digested cells were passed through a 70 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to remove the epithelial and undigested tissue fragments. The cell suspension was layered over a Ficoll-Paque gradient at 250 × g, 30 min (GE Healthcare UK Ltd., Buckinghamshire, UK). The obtained single-cell suspension in mononuclear cell layer was subsequently incubated with RPMI-1640 culture medium (Life Technologies Corporation) containing 10% fetal bovine serum (Life Technologies Corporation) at a concentration of 1 × 10⁵ cells/ml on a special coating plate for macrophage separation (MSP-P, Otsuka Assay Laboratories, Tokushima, Japan) in 5% CO₂ air at 37°C for 60 min (Andoh et al., 1991; Mizuno et al., 1994). After 60 min, the adherent cells were removed by a cell scraper (Naige Nunci International, NY, USA), washed twice and resuspended with RPMI-1640 culture medium (Life Technologies Corporation) containing 1% penicillin-streptomycin-glutamine (Life Technologies Corporation) and 10% fetal bovine serum (Life Technologies Corporation) at a concentration of 1 × 10⁶ cells/ml. The cells, thus obtained, were confirmed to be macrophages (Becot Dickinson, Carlsbad, CA, USA) at room temperature for 5 min. RPMI-1640 culture medium (Life Technologies Corporation) containing 1 mg/ml collagenase (Life Technologies Corporation) which has low protease activity, DNase I (100 μg/ml) (Roche Diagnostics, Basel, Switzerland), 1% penicillin–streptomycin–glutamine (Life Technologies Corporation) and 10% fetal bovine serum (Life Technologies Corporation) at 37°C for 60 min. The digested cells were passed through a 70 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to remove the epithelial and undigested tissue fragments. The cell suspension was layered over a Ficoll-Paque gradient at 250 × g, 30 min (GE Healthcare UK Ltd., Buckinghamshire, UK). The obtained single-cell suspension in mononuclear cell layer was subsequently incubated with RPMI-1640 culture medium (Life Technologies Corporation) containing 10% fetal bovine serum (Life Technologies Corporation) at a concentration of 1 × 10⁵ cells/ml on a special coating plate for macrophage separation (MSP-P, Otsuka Assay Laboratories, Tokushima, Japan) in 5% CO₂ air at 37°C for 60 min (Andoh et al., 1991; Mizuno et al., 1994). After 60 min, the adherent cells were removed by a cell scraper (Naige Nunci International, NY, USA), washed twice and resuspended with RPMI-1640 culture medium (Life Technologies Corporation) containing 1% penicillin-streptomycin-glutamine (Life Technologies Corporation) and 10% fetal bovine serum (Life Technologies Corporation) at a concentration of 1 × 10⁶ cells/ml. The cells, thus obtained, were confirmed to be macrophages microscopically and by α-Naphthyl butyrate esterase staining (MUTO PURE CHEMICALS Co., Ltd., Tokyo, Japan) routinely exceeded 95% (Chao et al., 2000). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and air in plastic plates (Becton Dickinson). After 24 h of pre incubation, interferon gamma (IFN-γ, 100 U/ml) (Roche) and lipopolysaccharide (LPS, 1 μg/ml) (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) were added as priming and triggering factors. Samples were collected after 0, 24 and 48 h, respectively. The cell culture samples were harvested by centrifugation with microtube at 12,000 × g for 10 min at 4°C and stored in lysing buffer at −20°C until use. The cell culture samples were sonicated in wash buffer using a sonifier (Branson Ultrasonic Corporation) at power M, level 4 for 5 min at 4°C. The samples were centrifuged at 12,000 × g for 30 min at 4°C, the supernatant fraction was collected and the protein concentration was measured by reading the absorbance at 562 nm using a plate reader (Thermo Scientific).

SDS–PAGE and western blot analysis

The supernatant fraction of tissue samples and cell culture samples were appropriately diluted with Laemmli sample buffer (62.5 mm Tris–HCl, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, pH 6.8) (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to SDS–PAGE according to Laemmli’s method (Laemmli, 1970). Into each lane of Criterion ready gel gels J (Bio-Rad) of 5–20%, 10 μg (decidual tissue samples) and 2 μg (cell culture samples) of protein, respectively, were applied together with a mid-range molecular weight standard marker (Bio-Rad). Electrophoresis was performed using SDS–PAGE cassettes (Bio-Rad), a power supply (Bio-Rad) and electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) (Bio-Rad). We performed western blot analysis using western blotting system (Bio-Rad), transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) to transfer the proteins. The membranes were blocked for 1 h at room temperature on a shaker (Taitec, Tokyo, Japan) with 5% skim milk (MEG MILK SNOW BRAND Co., Ltd., Tokyo, Japan) in PBS prior to primary antibodies. The following primary antibody and dilution were used: anti-cathepsin E antibody (H-40) sc-30055 [rabbit polyclonal immunoglobulin G (IgG)], 1/100 dilution (Santa Cruz Biotechnology, Inc., CA, USA). Anti-β-Actin antibody (Cell Signaling Technology, Inc., MA, USA) was used as loading control. As the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L)-HRP antibody (1/2000 dilution) (Bio-Rad) was used. Color development was achieved with Super Signal West Dura Extended Duration Substrate kit (Bio-Rad). The immunoreactive bands were semi-quantified using the software Science Lab 2005 Multi Gauge version 3.0 (Fuji Photo Film Co., Tokyo, Japan) and calculated with each membrane using controls. We ran two positive controls for each gel and converted the data. Data are shown as integrated optical density (OD), which were described previously (Kumagai et al., 2008).

Immunostaining

Immunohistology

Immediately after surgery, tissue samples were fixed in neutral buffered formaldehyde (‘Formalin neutral buffer water 10’, Ken-ei Seiyaku Ltd., Osaka, Japan) overnight. All samples were treated under the same conditions. The samples were dehydrated and embedded in paraffin, cut into 3 μm sections and collected on MAS-coated glass slides (Matsumani Glass Ind. Ltd., Osaka, Japan). After deparaffinization and rehydration through a series of xylene and ethanol, endogenous peroxidase activity was blocked by incubating the section with 3% H₂O₂ in methanol (Sigma-Aldrich Co.) for 15 min. After washing in PBS (Dulbecco’s Phosphate-Buffered Saline, Invitrogen., Inc., CA, USA) for 5 min three times, sections were incubated with primary antibodies diluted in TBS, 0.1% Tween, containing 3% bovine serum albumin (Invitrogen) overnight at 4°C. The following primary antibody and dilution were used: anti-cathepsin E antibody (H-40) sc-30055 [rabbit polyclonal immunoglobulin G (IgG)], dilution 1/250, concentration 0.8 μg/ml (Santa Cruz Biotechnology). Negative controls were made by rabbit serum, dilution 1/1000, concentration 5.2 μg/ml (Histofine, Nichirei, Tokyo, Japan). After washing in PBS for 5 min three times, sections were incubated with Histofine Simple Stain MAX-PO (peroxidase) (MULTI) (Nichirei) for 30 min at room temperature and washed in PBS for 5 min three times. Color development was performed using DAB (Nichirei). Sections were washed in water, and nuclei were counterstained with 1% methylgreen (Muto pure chemicals Co., Ltd, Tokyo Japan) for 10 min. Permanent specimens were made by dehydration through ethanol and xylene and sealing with ENTELLAN (Merck KGaA, Darmstadt, Germany).

Immunofluorescence

For immunofluorescence staining, after deparaffinization and rehydration through a series of xylene and ethanol, endogenous peroxidase activity was blocked by incubating the section with PBS containing 0.03% Triton X (Sigma-Aldrich Co.) and 5% BSA (Sigma-Aldrich Co.) for 1 h at room temperature. After washing in PBS containing 0.03% Triton X for 5 min three times, sections were incubated with primary antibodies diluted in PBS containing 0.03% Triton X, 5% BSA overnight at 4°C. For double staining, anti-cathepsin E antibody (Santa Cruz Biotechnology) and CD14 (Santa Cruz Biotechnology) or CD68 (Dako Cytomation, Glostrup, Denmark) were used as primary antibodies at 1:500 dilutions, respectively, with
incubation overnight at 4°C. The secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen) (1/1000 dilution) and Alexa Fluor 546 goat anti-rabbit IgG (H + L) (Invitrogen) (1/1000 dilution) were used with incubation at room temperature for 60 min. DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride, Roche Diagnostics) was used to detect nuclei. Permanent specimens were made with CC/Mount (DBS, CA, USA). Stained tissues were examined using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) and a confocal laser scanning microscope (LSM-510; Zeiss, Oberkochen, Germany). To detect Alexa Fluor 488 and Alexa Fluor 546 fluorescence simultaneously by confocal laser scanning microscope, Alexa Fluor 488 and Alexa Fluor 545 were excited at 488 nm (argon laser) and at 543 nm (He-Ne laser), and their fluorescence was observed with a band pass filter (500–530 nm) and a long pass filter (>560 nm), respectively.

Statistical analysis

To compare the number of littermates of CatE−/− mice, the cathepsin E activity in CM and the cathepsin E level in decidua of the patients with RM, nonparametric Mann–Whitney U-test was used. Data were expressed as median ± IQR (interquartile range). The level of significance used was \( P < 0.01 \). Generalized linear mixed models (Tuerlinckx et al., 2006) with cathepsin E level in decidual macrophages for the two factors (the time and stimulation with IFN-γ, LPS) were used to incorporate a parameter accounting for within-individual variations. All statistical analysis was performed on a personal computer using SPSS for Windows (version 14.0); SPSS, Chicago, IL, USA.

Results

CatE−/− mice show significantly smaller numbers of littermates and granulation tissue of uterus

Anecdotal observations indicated that CatE−/− mouse mating pairs were fertile, but the litter number was significantly smaller compared with wild-type mice. On average, wild-type females gave birth to 7.5 ± 1.38 litters, whereas CatE−/− females gave birth to only 4.83 ± 1.75 litters over the same time period \( (P < 0.001) \). The fetuses of CatE−/− mice varied in size (Fig. 1A). CatE−/− female mice showed uterine abnormality (Fig. 1B–D). The uterus of CatE−/− mice was swollen and had tumors in uterus horn. Histology of the tumor showed granulation tissue composed of fibrinous material and neutrophils at endometrial stroma. It contains necrotic tissue, bleeding, neutrophils and fibroblasts.

Cathepsin E activity in CM is significantly decreased in miscarriage group

We analyzed the correlation between activity of cathepsin E in CM of early pregnancy and their pregnancy outcome. Of a total of 21 patients, 11 (52%) miscarried subsequently. As shown in Table I, the patients’ background (age, number of miscarriages, gestational age at sampling)
was not significantly different between two groups. On the other hand, the average cathepsin E activity was significantly lower in patients who miscarried subsequently than in those who had a live birth ($P < 0.01$).

### The expression of cathepsin E in decidua is significantly lower in RM patients

Western blot analysis of decidual tissue in early pregnancy taken by surgical operation using anti-cathepsin E antibody showed a single band with molecular weight of 48 kDa, which corresponds to cathepsin E (Fig. 2). We semi-quantified the cathepsin E expression in deciduas of spontaneous miscarriage group ($n = 49$) and elective abortion group ($n = 24$). The results are shown in Table II. The expression of cathepsin E in decidua of RM patients was significantly lower than in the control group ($P < 0.01$).

### In decidua, the staining of cathepsin E is observed in CD14-positive cells and CD68-positive cells

By immunohistology, staining for cathepsin E was observed in the cytoplasm of cells in decidua of early pregnancy taken by dilatation and curettage (Fig. 3). No significant difference was observed in the staining.

### Table I Cathepsin E activity in cervical mucus in early pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>Live birth ($n = 10$)</th>
<th>Miscarriage ($n = 11$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$34 \pm 4.5$</td>
<td>$31 \pm 7$</td>
<td>n.s.</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>$2.5 \pm 0.5$</td>
<td>$3 \pm 1$</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gestational week</td>
<td>$5 \pm 1$</td>
<td>$5 \pm 0$</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cathepsin E activity</td>
<td>$0.2040 \pm 0.22$</td>
<td>$0.0995 \pm 0.06$</td>
<td>$&lt;0.01$</td>
</tr>
</tbody>
</table>

All values are median ± IQR. The cathepsin E activity in cervical mucus in early pregnancy was measured using cathepsin E-specific activity assay. The results are shown as median ± interquartile range ($n$: number of samples). The patient backgrounds (age, number of miscarriages, gestational week at sampling) of both groups were not significantly different. On the other hand, the average cathepsin E activity (U/mg) in cervical mucus was significantly lower in the miscarriage group compared with live birth group.

### Table II Comparison of semi-quantification of cathepsin E expression in decidual tissue (integrated OD).

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous miscarriage ($n = 49$)</th>
<th>Control ($n = 24$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$34 \pm 2.0$</td>
<td>$31 \pm 2.4$</td>
<td>n.s.</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>$2.9 \pm 1.5$</td>
<td>$0.0 \pm 0.0$</td>
<td>$&lt;0.05$</td>
</tr>
<tr>
<td>Number of live birth</td>
<td>$0.3 \pm 0.5$</td>
<td>$1.7 \pm 1.4$</td>
<td>$&lt;0.05$</td>
</tr>
<tr>
<td>Gestational week</td>
<td>$8.0 \pm 1.0$</td>
<td>$7.0 \pm 1.4$</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cathepsin E activity</td>
<td>$84.51 \pm 8.69$</td>
<td>$97.86 \pm 18.42$</td>
<td>$&lt;0.05$</td>
</tr>
</tbody>
</table>

Cathepsin E expression in decidua of spontaneous miscarriage group and control group (artificial abortion) was semi-quantified and compared, respectively. The patients and controls for this analysis are completely different from the cohort shown in Table I. The results of age, gestational week and cathepsin E activity are shown as median ± IQR (interquartile range); number of miscarriages and number of live birth are shown as mean ± SD ($n$: number of samples). The cathepsin E expression in decidua was significantly lower in the spontaneous miscarriage group compared with the control group.

### Figure 2

Western blot analysis of decidual tissue in early pregnancy using anti-cathepsin E antibody showed a single band with molecular weight of 48 Da, which corresponds to cathepsin E. Ten micrograms of protein from decidual tissue was used per lane. Anti-β-Actin antibody is provided as a loading control. P1–P3 is decidua of RM patients (spontaneous abortion), and C1–C3 is decidua of controls (artificial abortion). We semi-quantified each band for RM patients ($n = 49$) and controls ($n = 24$). The result is shown in Table II.

### Figure 3

Decidual tissue sections obtained from patients with recurrent miscarriage under the diagnosis of missed abortion (A) and from control (B) were stained with anti-cathepsin E antibody. Brown color represents the positive staining of anti-cathepsin E antibody. Cathepsin E immunoreactivity was observed in decidualized cells in decidual tissue. No remarkable difference was observed between patients and controls. Original magnification: $\times 400$. Scale bars show 100 μm.
pattern of any antibodies between RM patient and control (data not shown). Staining for cathepsin E was also detected in the cytoplasm of decidual macrophages collected from RM patients using macrophage separation plates (MSP-P) (Fig. 4).

We next analyzed the localization of cathepsin E in decidua taken from patients with RM by dilation and curettage under the diagnosis of missed abortion. By double-fluorescent labeling using antibodies directed against cathepsin E and CD14 or CD68, we examined the tissue samples using confocal laser scanning microscopy (LSM-510; Zeiss) (Fig. 5). In order to make clear the intracellular localization of cathepsin E, we examined the 3D image of cathepsin E in CD14 positive cells in decidua, using fluorescent microscopy (BZ-9000; Keyence). Cathepsin E immunoreactivity was detected as red granular dots in the cytoplasm, which is thought to be in the endosomal component, of CD14 or CD68 positive cells in decidua (Fig. 6).

Upon stimulation with LPS and IFN-γ, expression of cathepsin E is up-regulated in decidual macrophages

To assess the involvement of cathepsin E produced by decidual macrophages, the decidual macrophages obtained by dilation and curettage under the diagnosis of missed abortion were cultured with IFN-γ and LPS as priming and triggering factors, and we measured the cathepsin E

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**Figure 4** Immunocytochemistry was performed on primary decidual macrophages taken and cultured from patients with consecutive miscarriage on chamber slides. The cells were stained with anti-CD68 antibody (A) and anti-cathepsin E antibody (B). Using MSP-P, CD68 positive cells were collected from decidua of RM patients. Brown color represents the positive staining of each antibody. n (number of experiments) = 5. Original magnification: ×400. Scale bar shows 50 μm.

**Figure 5** Decidual tissue sections obtained from patients with recurrent miscarriage under the diagnosis of missed abortion were double-stained with anti-cathepsin E antibody and anti-CD14 or anti-CD68 antibody using laser scanning confocal microscopy. (Upper panel) Cathepsin E (red) was seen in the cytoplasm of the decidual cells. CD14 (green) was seen in the membrane of the decidual cells. (Lower panel) CD68 (green) was seen in the cytoplasm of the decidual cells. Yellow color represents the colocalization of Cathepsin E (red) and CD14 or CD68 (green). n (number of experiments) = 5.
expression by western blot analysis using anti-cathepsin E antibody (red) and anti-CD14 antibody (green). For evaluation of intracellular localization of cathepsin E, the 3-dimensional observation of a decidual macrophage using fluorescent microscopy (BZ-9000; Keyence) was done. Upper panels show 'a bird's eye view'. Lower panels, X, Y, and Z, show each view from X-axis, Y-axis, and Z-axis, respectively. Arrows show the bottom of plates. The nuclei were stained with DAPI (blue). Yellow color represents the co-localization of Cathepsin E and CD14. Object lens magnification: ×100.

Discussion

We have reported that imbalance of cathepsin-cystatin system plays an important role in miscarriage (Nakanishi et al., 2005). Previous studies have revealed a substantial role of cathepsin E in immune defenses, such as host defense against tumor cells (Kawakubo et al., 2007), enhancement of immune defense against infection and a preventive role for development of atopic dermatitis (Tsukuba et al., 2003). We have shown that Cathepsin-deficient (CatE−/−) female mice display reduced litter number compared with wild-type controls. Our data suggested that cathepsin E deficiency is not lethal, but has important roles in reproduction. Moreover, CatE−/− mice showed inflammatory changes in the area of endometrial stroma of the uterus. However, there are few studies which evaluated the correlation between cathepsin E expression and miscarriage. Based on our hypothesis that cathepsin E produced by decidua correlates with miscarriage, we evaluated for the first time the protein expression of cathepsin E in human decidua, especially in decidual macrophages from patients with RM.

We compared the cathepsin E activity in CM with pregnancy outcome prospectively. In the miscarriage group, cathepsin E activity in CM was significantly lower than in the controls, which suggests a correlation between lower secretion or activation of cathepsin E in human uterus and the etiology of spontaneous miscarriage. Based on our hypothesis that cathepsin E produced by decidua correlates with miscarriage, we evaluated for the first time the protein expression of cathepsin E in human decidua, especially in decidual macrophages from patients with RM.

Cathepsin E has been reported to display limited tissue distribution, mainly present in cells of the immune system such as macrophages, microglia, dendritic cells (Yamamoto et al., 2012) and lymphocytes (Sakai et al., 1989). Until now, there has been no report revealing the existence of cathepsin E in human uterus in early pregnancy. In order to clarify the existence and localization of cathepsin E in human uterus, we used western blot analysis and immunohistochemistry. The results showed the expression of cathepsin E in human decidua, including decidual macrophages (Shin et al., 2007). The intracellular localization of cathepsin E has

Figure 6 Decidual tissue sections obtained from patients with recurrent miscarriage under the diagnosis of missed abortion and control (data not shown) were double-stained with anti-cathepsin E antibody (red) and anti-CD14 antibody (green). For evaluation of intracellular localization of cathepsin E, the 3-dimensional observation of a decidual macrophage using fluorescent microscopy (BZ-9000; Keyence) was done. Upper panels show 'a bird's eye view'. Lower panels, X, Y, and Z, show each view from X-axis, Y-axis, and Z-axis, respectively. Arrows show the bottom of plates. The nuclei were stained with DAPI (blue). Yellow color represents the co-localization of Cathepsin E and CD14. Object lens magnification: ×100.

Figure 7 Decidual macrophages were collected from patients with recurrent miscarriage and incubated with or without IFN-γ (100 U/ml) and LPS (1 μg/ml). The change in cathepsin E expression of cytosolic fractions of decidual macrophages was compared at time 0, time24, time48, semi-quantified with SDS–PAGE, western blotting. Anti-β-Actin antibody is used as a loading control. The cathepsin E expression of decidual macrophage was decreased when incubated with IFN-γ and LPS (open square) compared without IFN-γ and LPS (closed triangle). Closed triangle and open square mean mean value. n (number of experiments) = 6.
been reported to vary according to cell type (Yamamoto et al., 2012). In antigen-presenting cells (APCs), such as macrophages, microglia and dendritic cells, the enzyme is mainly localized in the endosomal compartments as a mature form (Yamamoto et al., 2012). In our study, we showed for the first time that staining of cathepsin E was in the cytoplasm of CD14 or CD68 positive cells in human decidua using confocal laser scanning microscopy and fluorescent 3D analysis.

Next, to assess the involvement of cathepsin E produced by decidual macrophages, we separated and stimulated decidual macrophages by IFN-γ and LPS, and semi-quantified the cathepsin E expression in cell lysates using SDS–PAGE and western blotting. IFN-γ and LPS treatment decreased the intracellular levels of cathepsin E in decidual macrophages. We tried to measure the cathepsin E level in culture supernatant, but it could not be detected because the cathepsin E level was lower than the detectable range. Previous data using a mouse model showed that upon stimulation with IFN-γ or LPS, the activity levels of cathepsin E in the cell lysates of mouse macrophages were markedly decreased, but those in the culture media were largely increased, which was due to the enhanced secretion of the enzyme to the culture media (Yanagawa et al., 2006). Our study on humans also indicated that cathepsin E is up-regulated and secreted by IFN-γ and LPS treatment in human decidual macrophages in a time-dependent manner.

Moreover, it is reported that activity of cathepsin E in rat uterine tissue is increased by administration of estradiol and progesterone (Gladson et al., 1998). Since estradiol and progesterone increase dramatically in the first trimester of pregnancy in human, cathepsin E may be activated by such hormonal changes.

In human decidual tissue, CD14+ decidual macrophages are abundant and they are the most important professional APCs in the decidua (Gardner and Moffett, 2003). Since decidual macrophages express MHC class II and are closely associated with extravillous trophoblast invasion. Moreover, recently, pro-inflammatory and anti-inflammatory macrophages induced from peripheral monocytes by different stimuli were named M1 or M2 macrophages in line with the Th1/Th2 paradigm (Martinez et al., 2006). Atopic dermatitis is known to be a chronic inflammatory skin disease characterized by the predominance of Th2-type cytokines (Murphy and Reiner, 2002; Finkelman et al., 2004), and their expression of cathepsin E in patients with atopic dermatitis was significantly lower compared with healthy subjects (Tsukuba et al., 2003). In unexplained RM, it has been reported that the decreased immunosuppressive function of regulatory T cells resulted in an imbalance of Th1/Th2 and abnormality of maternal-fetal immuno-tolerance (Jiang et al., 2009). Because cathepsin E plays a nonredundant role in the class II MHC Ag processing pathway within dendritic cells (Chain et al., 2005), cathepsin E might play a substantial role in appropriate function of M1, pro-inflammatory type of decidual macrophages to maintain appropriate M1/M2 balance in early pregnancy. The data obtained in our study showed that in RM patients, cathepsin E expression is significantly lower compared with controls, which suggests that patients with RM may also have an M1/M2 imbalance which interrupts the normal maternal-fetal immuno-tolerance and appropriate trophoblast invasion.

In conclusion, we have demonstrated for the first time the existence of cathepsin E in human decidual tissue, and activity of cathepsin E produced by decidual macrophages may play an important role in the establishment of early pregnancy.

We suggest that one promising future clinical application could be measuring cathepsin E using CM for prognosis of pregnancy outcome or evaluation of therapy.

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Authors’ roles

S.G. and Y.O. carried out experiments, collection and assembly of data, data analysis, manuscript writing and interpretation. N.S. involved in conception and design. A.Y., T.K. and K.Y. carried out experiments and contributed to provision of experiment material. M.S.O. involved in conception and design.

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