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ORIGINAL RESEARCH

Role of cathepsin E from decidual macrophages in 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 Fluorescence-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, W-B 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 / deciduas / 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; Sugiura-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). Recent reports suggested that a variety of immunological mechanisms may be involved in RM involving decidual macrophages (Jin *et al.*, 2009) serum macrophage-colony-stimulating factor (Katano *et al.*, 1997), and cervical interleukins, IL-6 and IL-8, inflammatory cytokines produced by macrophages, proposed to have predictive value for cases of RM (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 placental 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; Keliher *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 administrated 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 littermates 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 (Katano *et al.*, 2013), determination of antiphospholipid antibodies (aPLs) including lupus anticoagulant by activated partial thromboplastin time, diluted Russel viper venom time and β_2 glycoprotein I-dependent anticardiolipin antibody method (Ogasawara *et al.*, 1996), blood tests of free testosterone (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 $12\,000 \times g$ 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, avoided 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-NH_2 (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 ethyleneglycoltetraacetic acid (EGTA), 10 mM 2-mercaptoethanol, 10 μM p-4-amidinophenylmethane sulfonylfluoride 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 Fisher 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 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 m 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 1×10^5 cells/ml on a special coating plate for macrophage separation (MSP-P, Otsuka Assay Laboratories, Tokushima, Japan) in 5% CO_2 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 (Nalge Nunc 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 1×10^5 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 gradient 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 revising each membrane using controls. We ran two positive controls for each gel and convert 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 (Matsunami 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% H_2O_2 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 14.0J; 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 1, the patients' background (age, number of miscarriages, gestational age at sampling)

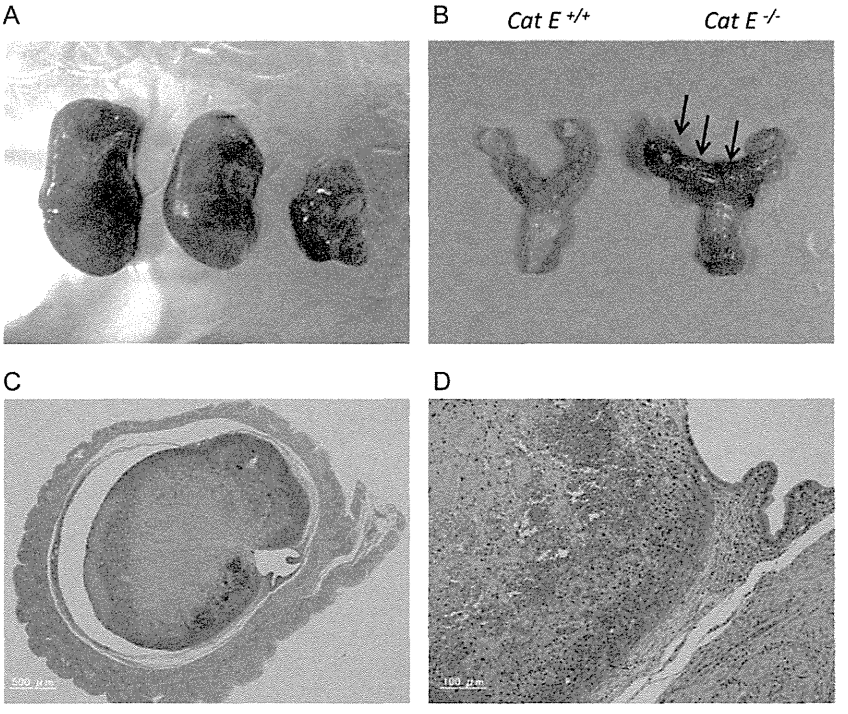

Figure 1 The female cathepsin E-deficient ($CatE^{-/-}$) mice were kept apart from the male mice over 25 days. Fetuses still remained in the uterus. (**A**) The fetuses of $CatE^{-/-}$ mice varied in size. (**B**) The uterus of $CatE^{-/-}$ mice was swollen and had tumors in uterus horn (arrows). Left is the uterus of wild-type mouse. (**C**) Histology of the tumor showed granulation tissue composed of fibrinous material and neutrophils in the endometrial stroma. Scale bar shows 500 μm. (**D**) High-power field of granulation tissue. It contains necrotic tissue, bleeding, neutrophils and fibroblasts. Scale bar shows 100 μm.

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 ($P < 0.01$).

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

	Live birth ($n = 10$)	Miscarriage ($n = 11$)	P
Age	34 ± 4.5	31 ± 7	n.s.
Number of miscarriages	2.5 ± 0.5	3 ± 1	n.s.
Gestational week	5 ± 1	5 ± 0	n.s.
Cathepsin E activity	0.2040 ± 0.22	0.0995 ± 0.06	<0.01

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.

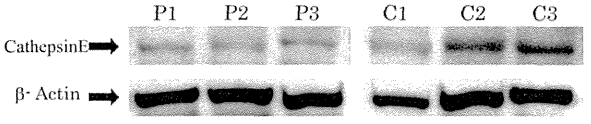

Figure 2 Western blot analysis of decidual tissue in early pregnancy using anti-cathepsin E antibody showed a single band with molecular weight of 48 Da, which corresponds to cathepsin E. Ten micrograms of protein from decidual tissue was used per lane. Anti-β-Actin antibody is provided as a loading 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.

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

	Spontaneous miscarriage ($n = 49$)	Control ($n = 24$)	P
Age	34 ± 2.0	31 ± 2.4	n.s.
Number of miscarriages	2.9 ± 1.5	0.0 ± 0.0	<0.05
Number of live birth	0.3 ± 0.5	1.7 ± 1.4	<0.05
Gestational week	8.0 ± 1.0	7.0 ± 1.4	n.s.
Cathepsin E activity	84.51 ± 8.69	97.86 ± 18.42	<0.05

Cathepsin E expression in decidual tissue 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.

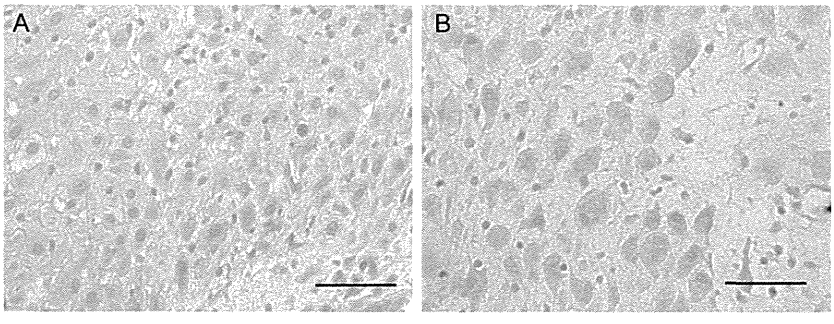

Figure 3 Decidual tissue sections obtained from patients with recurrent miscarriage under the diagnosis of missed abortion (**A**) and from control (**B**) were stained with anti-cathepsin E antibody. Brown color represents the positive staining of anti-cathepsin E antibody. Cathepsin E immunoreactivity was observed in decidualized cells in decidual tissue. No remarkable difference was observed between patients and controls. Original magnification: ×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 deciduas 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

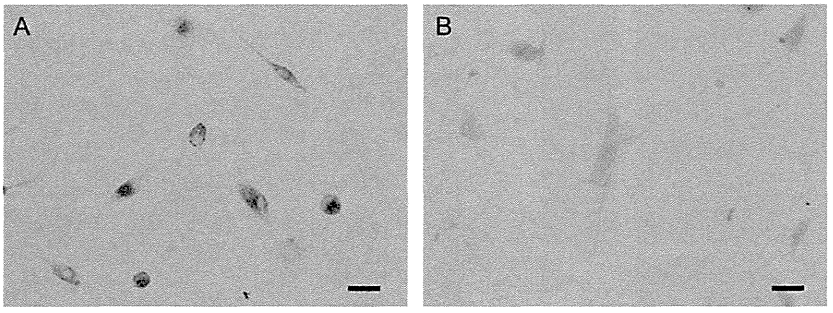

Figure 4 Immunocytochemistry was performed on primary decidual macrophages taken and cultured from patients with consecutive miscarriage on chamber slides. The cells were stained with anti-CD68 antibody (**A**) and anti-cathepsin E antibody (**B**). Using MSP-P, CD68 positive cells were collected from 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.

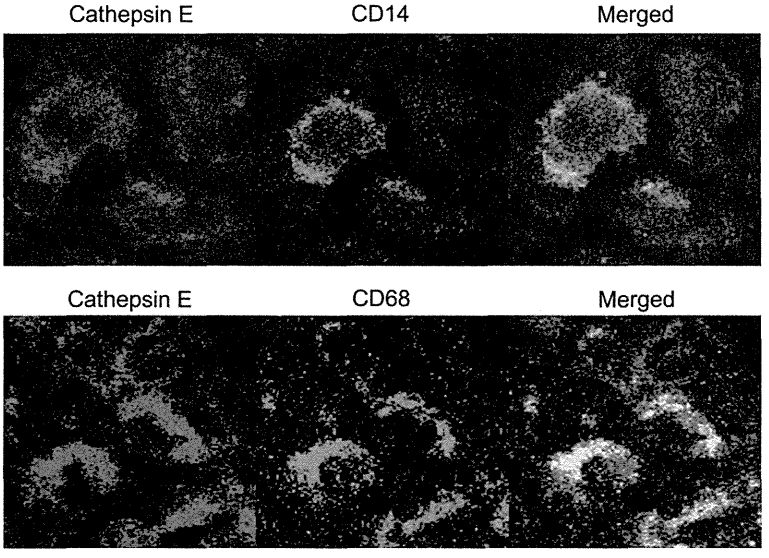

Figure 5 Decidual tissue sections obtained from patients with recurrent miscarriage under the diagnosis of missed abortion were double-stained with anti-cathepsin E antibody and anti-CD14 or anti-CD68 antibody using laser scanning confocal microscopy. (Upper panel) Cathepsin E (red) was seen in the cytoplasm 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.

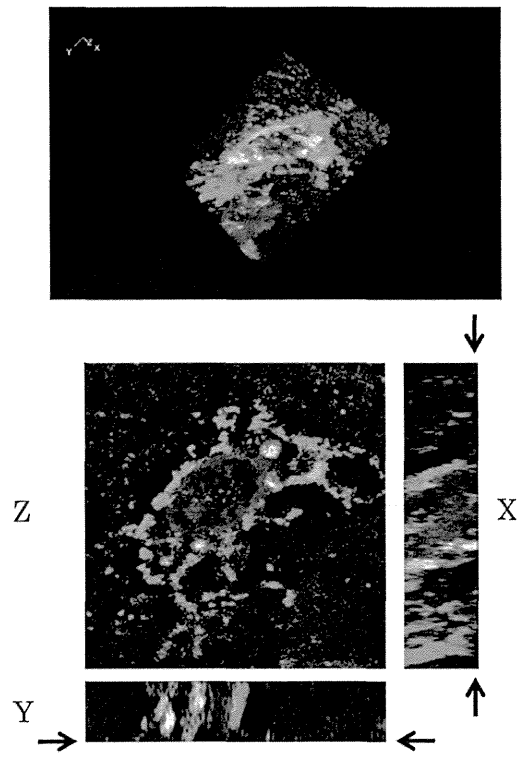

Figure 6 Decidual tissue sections obtained from patients with recurrent miscarriage under the diagnosis of missed abortion and control (data not shown) were double-stained with anti-cathepsin E antibody (red) and anti-CD14 antibody (green). For evaluation of intracellular localization of cathepsin E, 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.

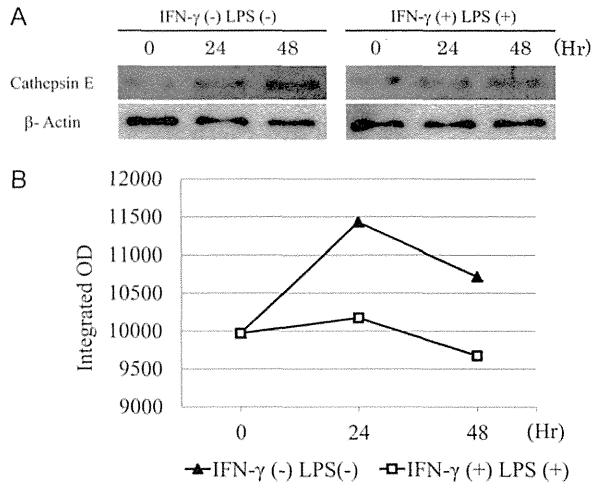

Figure 7 Decidual macrophages were collected from patients with recurrent miscarriage and incubated with or without IFN-γ (100 U/ml) and LPS (1 μg/ml). The change in cathepsin E expression of cytosolic fractions of decidual macrophages was compared at time 0, time24, time48, semi-quantified with SDS–PAGE, 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.

expression by western blot analysis using anti-cathepsin E antibody. As shown in Fig. 7A, a single band with molecular weight of 48 kDa, which corresponds to cathepsin E, was detected in decidual macrophages. Semi-quantification of cathepsin E expression in decidual macrophages stimulated by IFN-γ and LPS and unstimulated decidual macrophages is shown in Fig. 7B. The expression of cathepsin E in cell lysates of decidual macrophages was significantly lower in the stimulated group after 24 and 48 h of stimulation.

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. Moreover, it might have predictive potential for pregnancy outcome in early pregnancy.

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. Semi-quantitative analysis revealed a lower expression of cathepsin E in decidua of RM patients, which implies a correlation between lower expression of cathepsin E in human decidua and the etiology of spontaneous miscarriage (Shin *et al.*, 2007). The intracellular localization of cathepsin E has

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 (Bulmer *et al.*, 1988), they may have an important role in the establishment of fetal–maternal immune tolerance and the control of the 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 functions 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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Management of recurrent miscarriage

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Abstract

Recurrent miscarriage is classically defined as three or more consecutive pregnancy losses. Many researchers have now revised this definition to two or more pregnancy losses because of the recent increase in the prevalence of childless couples. Established causes of recurrent miscarriage are antiphospholipid antibodies, uterine anomalies and abnormal chromosomes in either partner, particularly translocations. Antiphospholipid syndrome is the most important treatable cause of recurrent miscarriage. However, it is not yet established as to what kind of testing should be conducted in patients with recurrent pregnancy loss. Standardization of tests for antiphospholipid antibodies is needed. On the other hand, embryonic aneuploidy is the most frequent cause of recurrent miscarriage. Chromosome analysis of the embryo is important, because it has good predictive value for subsequent live birth. It is not necessary to give any medications for unexplained cases of recurrent miscarriage, and provision of psychological support may be the most important to encourage the couples to continue to conceive until a live birth results.

Key words: antiphospholipid antibodies, embryonic karyotype, recurrent miscarriage, translocation, uterine anomalies.

Causes of Recurrent Miscarriage

Recurrent miscarriage (RM) is classically defined as three or more consecutive pregnancy losses. However, many researchers have now revised the definition to two or more pregnancy losses, because of the recent increase in the prevalence of childless couples. The estimated frequencies of three or more and two or more consecutive pregnancy losses are 0.9% and 4.2%, respectively, in the Japanese general population.[1] For the present review, RM is defined as two or more consecutive pregnancy losses.

Antiphospholipid antibody (aPL) syndrome (APS), uterine anomalies and abnormal chromosomes in either partner are established causes of RM.[2–4] Only about 30% of cases of RM have an identifiable cause (Fig. 1a),[3] and it is well-known that the cause remains indeterminate in over a half of the cases.[5] According to one recent study, the abnormal embryonic karyotype was found in 41.1% of subjects in whom no conventional causes of miscarriage could be identified (Fig. 1b).[6] Therefore, the percentage of cases with RM of truly unexplained cause may not exceed 24.5%.

The reported incidence of APS is 5–15%.[5] However, the references quoted in this review were published before the International Criteria for APS were published.[7] In our previous study, the incidences of APS, translocation and major uterine anomalies were 4.5%, 3.2% and 4.5%, respectively.[3,4]

The clinical tests recommended for identifying the cause of RM are shown in Table 1. Endocrine disturbances have also been postulated to cause RM, but few randomized controlled trials or cohort studies reporting endocrine disturbances as a cause have withstood scrutiny. We examined blood tests for hypothyroidism and diabetes mellitus and ultrasonography for polycystic ovarian syndrome to detect endocrine causes of RM. However, Branch *et al.* do not recommend blood tests

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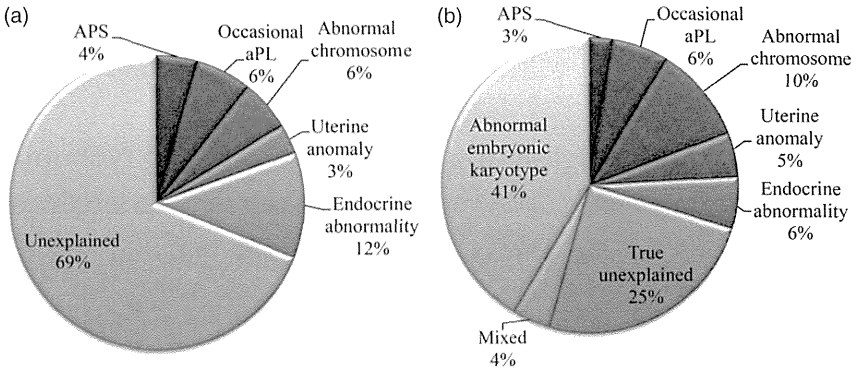

Figure 1 Comparison of the distribution of causes in (a) the 1676 subjects in our previous study[3] and (b) our 482 patients with recurrent miscarriage (RM), including those with an abnormal embryonic karyotype.[6] aPL, antiphospholipid antibody; APS, aPL syndrome.

Table 1 Tests recommended for patients with recurrent miscarriage

Recommended tests	
Antiphospholipid antibody syndrome	Lupus anticoagulant using both activated partial thromboplastin time and Russel viper venom time, anticardiolipin antibodies or anti-b2-glycopritein I antibodies
Uterine anomaly	Ultrasonography and hysterosalpingography, or hysteroscope
Abnormal chromosome	Chromosome analysis of both father and mother
Abnormal embryonic karyotype	Chromosome analysis of the products of conception
Endocrine abnormality	Other tests for diabetes or hypothyroidism if suggested by history or physical examination

for hypothyroidism, diabetes mellitus or thrombophilia as potential causes of RM.[5] It has not been established whether thrombophilia, immune dysfunction, infection and psychological stress may contribute to RM.

APS

APS is the most important treatable cause of RM. The clinical criteria for the diagnosis of APS include:

1 Three or more consecutive unexplained miscarriages before the 10th week of gestation.
2 One or more unexplained death of a morphologically normal fetus at 10 weeks of gestation or later.
3 One or more premature births of a morphologically normal fetus at 34 weeks of gestation or earlier, associated with severe pre-eclampsia or placental insufficiency.[7]

According to the new international criteria, patients can be diagnosed as having APS when positivity for lupus anticoagulant (LA) by activated partial thromboplastin time (aPTT) or dilute Russel viper venom time (RVVT), and/or anticardiolipin antibodies (aCL) or anti-β2glycoprotein I (β2GPI) antibodies are persistent for 12 weeks. The 99th percentile in healthy controls is recommended as the cut-off for the antiphospholipid tests. Combined low-dose aspirin plus heparin treatment is accepted as the standard treatment for patients with APS.[8,9]

We conducted a prospective study to examine whether a positive test result for β2GPI-dependent aCL might predict adverse pregnancy by 10 weeks of gestation in 1125 pregnant women without complications; results obtained using a cut-off value of 1.9 (99th percentile in healthy volunteers) were found to have a predictive value for intrauterine fetal death, intrauterine growth restriction and pre-eclampsia.[10] However, in the study, it could not be ascertained whether β2GPI-dependent aCL might have been of predictive value for early miscarriage, because the sampling was conducted only at about 10 weeks of gestation. Furthermore, we established a test for LA by 5x-diluted aPTT with the mixing test (LA-aPTT) and proved that treatment could improve the subsequent live birth in patients with a positive test result.[11]

As it is well recognized that the true antigens of aPL are not phospholipids, but phospholipid-binding plasma proteins, such as β2GPI, prothrombin, kininogen, protein C and protein S, many kinds of antiphospholipid tests are available;[12,13] in fact, there are over 10 commercially available methods in Japan. The antiphosphatidylethanolamine antibodies (aPE) method has been the most frequently used in Japan because of the positive rate, although its clinical significance remains unclear.[14] We identified a population

with aPE immunoglobulin (Ig)G that was distinct from that of patients showing positive test results for the conventional antiphospholipid tests, and treatment could not improve the live-birth rate in patients with aPE IgG who did not show a positive test result of LA-aPTT.[15] These results suggest that standardization is needed for detecting obstetric APS to treat RM. We recommend both LA-aPTT by StaClot and LA-RVVT, but not the tests for anticardiolipin IgG or IgM, because LA-aPTT and LA-RVVT were found to have obstetric significance and the distribution of the results differed between the two (submitted).

In our study, the frequency of antinuclear antibody (ANA) in 225 RM patients was significantly higher than that in 740 normal pregnant controls; however, there was no significant difference in the subsequent miscarriage rate between the ANA-positive and ANA-negative cases.[16]

We usually carry out LA-aPTT and LA-RVVT, and also β2GPI-dependent aCL clinically. The likelihood that at least one of the three tests is positive is 10.7%, and in 4.5%, the positive finding is sustained for 12 weeks until APS is diagnosed. Patients with persistent aPL should be treated by combined low-dose aspirin and heparin therapy during pregnancy, and a live birth can be expected in 70–80% of the patients treated thus.[8,9] Precise calculation of the gestational weeks can be made from the basal body temperature chart. Combined treatment with low-dose aspirin and heparin calcium at 10 000 IU/day (twice a day) should be started from 4 weeks of gestation. We discontinue aspirin by 35 weeks of gestation and continue heparin until the onset of labor.

It is not yet clear whether patients with occasional aPL should be treated. We found that the live-birth rate in 52 patients with occasional aPL treated with aspirin was significantly higher than that in 672 patients with unexplained RM who received no medication.[17] We concluded that aspirin is also useful in patients with occasional aPL, but not APS, diagnosed on the basis of the test results of LA-aPTT, LA-RVVT and β2GPI-dependent aCL.

Congenital Uterine Anomaly

The estimated frequency of congenital uterine anomalies in women with a history of RM is 1.8–37.6%. The wide range can largely be explained by the methods of patient selection and criteria for diagnosis.[18–20] The associations between arcuate uterus and RM, and between anomalies and infertility remain controversial.

Affected patients may be offered the option of surgery in an attempt to restore the uterine anatomy. While this may provide hope that the operations would increase the rate of successful pregnancies, to the best of our knowledge, there have been no prospective studies comparing the pregnancy outcomes between cases of RM with uterine anomalies treated and not treated by surgery.

A comparison of the subsequent live-birth rate after two or more miscarriages in 1676 patients with and without congenital uterine anomalies revealed that 54 (3.2%) had major uterine anomalies. Of these, 59.5% of patients (25/42) with a bicornuate or septate uterus had a successful first pregnancy after the examination, while the corresponding rate for women with normal uteri was 71.7% (1096/1528, $P = 0.084$). There was no significant difference in the cumulative live-birth rates (78.0% and 85.5%) between the two groups during the follow-up period.[3] On the other hand, rates of abnormal chromosome karyotype in the aborted concepti were 15.4% (2/13) and 57.5% (134/233) in the two groups, respectively, the latter being significantly higher. The defect/cavity ratio was also significantly higher in the subsequent miscarriage group than that in the live birth group ($P = 0.006$). Because of a value of 0.8 for the area under curve of the receiver–operator curve, major uterine anomalies clearly have a negative impact on the reproductive outcome in women with RM, being associated with a higher risk of further miscarriage with a normal embryonic karyotype. The defect/cavity ratio was found to have predictive value for further miscarriage in RM patients. This information should be given to the patients before any surgery is undertaken. A prospective case–control study is necessary to compare the live-birth rates between patients with and without surgery, also taking into consideration the infertility rate.

Abnormal Chromosomes in Either Partner

Based on a review of the data of 22 199 couples, De Braekeler and Dao concluded that the rate of chromosomal structural rearrangements in couples with a history of two or more spontaneous abortions was 4.7%.[21]

We conducted the first case–control study of 1284 couples to examine whether translocations constituted

a risk factor for RM.[4] Our findings indicated a successful pregnancy rate of 31.9% (15/47) in the first pregnancy after ascertainment of the carrier status, which is much less than that reported in cases with normal chromosomes (71.7%, 849/1184), and a cumulative successful pregnancy rate of 68.1% (32/47). We concluded that the prognosis of RM patients with reciprocal translocations is poor, given that the study was conducted over a 17-year period, and included severe cases with a history of 10 to 13 miscarriages.

Franssen *et al.* reported cumulative successful pregnancy rates in RM patients with reciprocal translocations, Robertsonian translocations and a normal karyotype of 83.0%, 82.0% and 84.1%, respectively, based on a prospective case–control study.[22] They concluded that the chance of having a healthy child was as high as that in non-carrier couples, despite the higher risk of miscarriage.

The live-birth rates with preimplantation genetic diagnosis (PGD) per oocyte retrieval in reciprocal translocation carriers[23–26] are reported to be comparable to or sometimes lower than those[4,27,28] with a subsequent first natural conception (Table 2). It is difficult, however, to simply compare *in vitro* fertilization (IVF) plus PGD and natural conception in translocation carriers. Information on the live-birth rate in the subsequent first pregnancy and time-based, not cycle-based, cumulative pregnancies after IVF plus PGD or natural conception is necessary.

The Japan Society of Obstetrics and Gynecology ruled for ethical reasons that PGD can be permitted only in cases with extremely severe genetic disease and RM with translocation. It is important that the final decision is made by couples with RM, not physicians and scientists. Thus, they should be fully informed of the advantages and disadvantages of both PGD and natural pregnancy. Genetic counseling and explanation of the live-birth rate with natural conception should be provided before any subsequent pregnancy after ascertainment of the carrier status.

Abnormal Embryonic (Fetal) Karyotypes in Unexplained Recurrent Miscarriage

Embryonic aneuploidy is the most important cause of miscarriage before 10 weeks of gestation and our previous study showed that 41% of women with RM had an abnormal embryonic karyotype.[6] However, this cannot be conclusive, because aborted concepti are seldom karyotyped clinically. A recent microarray comparative genomic hybridization approach indicated that about 80% of all sporadic spontaneous abortions are caused by an abnormal embryonic karyotype. In our previous study, the live-birth rate decreased and the normal embryonic karyotype increased according to the number of previous miscarriages.[29] The live-birth rate of patients with a previous abnormal embryonic karyotype was significantly higher, which suggests that the embryonic karyotype can be a good predictor of subsequent success.

Preimplantation genetic screening for aneuploidy could not improve the live-birth rate in women with unexplained RM.[30] Paternal immunization, or treatment with low-dose aspirin and heparin had no effect of improving the live-birth rate in women with unexplained RM.[31,32] However, patients with unexplained RM also desire to receive medication. The reported subsequent live-birth rates in patients with two, three, four and five miscarriages are 80%, 70%, 60% and 50%,

Table 2 Comparisons of pregnancy outcomes between PGD and natural pregnancy in reciprocal translocation carriers with a history of recurrent miscarriage

	PGD					Natural pregnancy		
	Lim[23]	Otani[24]	Feyereisen[25]	Fischer[26]	Sugiura-Ogasawara[27]	Stephenson[28]	Multicenter in Japan[27]	Franssen[22]
No. patients	43	29	35	192	47	20	46	157
Age	31.5	32.7		34	29.1		31	
Previous miscarriage		3.4					3.1	
Cycle to OR	59	36	81	272				
Positive hCG	22	17		69				
Delivery	14	17	5	60	15	13	29	131
Birth rate (%)	32.6	58.6	14.3	31.3	31.9	65	63	
OR (%)	23.7	47.2	6.2	22.1				
Cumulative live-birth rate (%)					68.1	90		83

hCG, human chorionic gonadotrophin; OR, odds ratio; PGD, preimplantation genetic diagnosis.

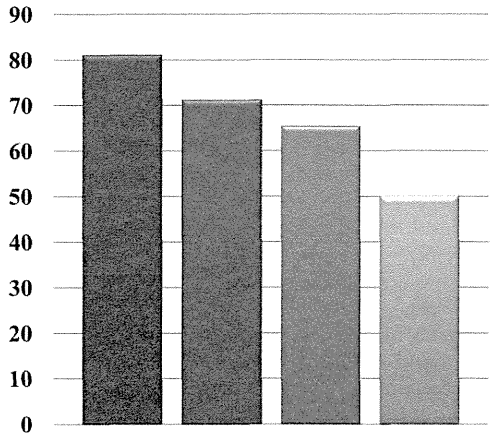

Figure 2 The live-birth rate increased with the number of previous miscarriages. ■, 2 times; ■, 3 times; ■, 4 times; ■, 5–6 times.

respectively (Fig. 2).[33] It is important to make the patients aware that no medications have been shown to improve upon the live-birth rate shown above.

Recently, existence of an association between many kinds of polymorphisms and RM has been reported. The frequency of SNP5 of the annexin A5 gene was found to be higher in women with RM than that in fertile women in our cross-sectional study.[34] However, there was no difference in the subsequent live-birth rate between patients with and without the mutation in our cohort study.[34] This means that a single mutation with a small odds ratio exerts little influence on miscarriage and that several kinds of mutations existing together induce susceptibility to RM.

Several couples in our experience gave up trying to conceive after RM, because they had the misconception that it would be impossible for them to have a living baby. Psychological support with tender loving care may be the most important factor to encourage such couples to continue to conceive until a live birth results.

Disclosure

None of the authors have any conflicts of interests to report.

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Genotyping analyses for polymorphisms of *ANXA5* gene in patients with recurrent pregnancy loss

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Objective: To investigate whether polymorphisms at the promoter or 5′-untranslated region of annexin A5 gene (*ANXA5*) influence miscarriage.
Design: Case-control study and nested case-control study.
Setting: Hospitals.
Patient(s): A total of 264 patients with two to nine recurrent pregnancy losses (RPLs) and 195 fertile control subjects.
Intervention(s): None.
Main Outcome Measure(s): The frequency of six single-nucleotide polymorphisms (SNPs) of the *ANXA5* gene in RPL patients versus control subjects, and subsequent live birth rate with and without risk alleles in RPL patients.
Result(s): The minor allele was significantly more frequent in RPL patients than in control subjects for SNP5 (rs1050606). The live birth rates of patients with and without risk alleles of SNP5 were 84.0% and 84.3%, respectively, after excluding cases with abnormal embryonic karyotype, with no significant difference.
Conclusion(s): The variations with the *ANXA5* gene upstream region, especially SNP5, were confirmed to be risk factors of RPL. However, presence/absence of the *ANXA5* risk allele did not have any predictive effect for subsequent pregnancy outcome. This was the first study indicating the influence of *ANXA5* SNP5 for pregnancy outcome. (Fertil Steril® 2013;100:1018–24. ©2013 by American Society for Reproductive Medicine.)
Key Words: Annexin A5, single-nucleotide polymorphisms, recurrent pregnancy loss, cohort

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Established causes of recurrent pregnancy loss (RPL) include antiphospholipid antibody syndrome (APS), uterine anomalies, and abnormal chromosomes, particularly translocations, in either partner (1–3). However, according to earlier reports, in about one-half of the cases seen at research centers, the cause remains unexplained despite conventional examinations conducted to identify the cause (4, 5). Recently, we found that an abnormal embryonic karyotype was the most frequent cause of RPL, accounting for as many as 41% of the cases (6).

APS, or acquired thrombophilia, is the only treatable cause of RPL, with combined low-dose aspirin and heparin treatment having been shown to improve the live birth rate in patients with APS (7, 8). Heritable thrombophilia has been reported to be the cause in a majority of other cases of pregnancy loss of uncertain cause

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(9, 10). However, no association has been established between hereditary thrombophilia and RPL (11).

Annexin A5 (placental anticoagulant protein, encoded by the *ANXA5* gene) normally occurs on the placental villi, and its expression appears to be decreased in the presence of antiphospholipid antibodies (aPL) (12). Annexin A5 functions as an inhibitor of coagulation by its ability to bind to the anionic phospholipids exposed on the surface of platelets (13). It is abundantly expressed in the normal placenta (14), and the encoding gene possesses a complex promoter region that is subject to intricate regulation (15). It has been shown that single-nucleotide polymorphisms (SNPs) in the promoter region of the *ANXA5* gene are significantly associated with RPL, and that women with the M2 haplotype have a 2.42-fold higher risk of pregnancy loss than noncarriers (16). More recently, it has been shown that an SNP in the 5′-untranslated region of the *ANXA5* gene (SNP5: −302T>G) serves as a major risk determinant of RPL in Japanese women (17).

In the present study, we further investigated six *ANXA5* gene SNPs in the upstream region in a cohort study of Japanese women with RPL and merged the findings with previously published reports (17) for univariate analyses. Because data on the combined effect of maternal age and genetic risk factors are still lacking, we also conducted a cohort study to examine, in relation to several factors, whether a subsequent pregnancy would result in further loss or normal delivery. This was the first cohort study to investigate *ANXA5* gene polymorphism as a molecular marker of RPL.

MATERIALS AND METHODS

Patients

We analyzed the data of 264 Japanese women with a history of unexplained RPL (defined as a history of two or more pregnancy losses) who were recruited from Nagoya City University Hospital from June 2007 to November 2012. All patients underwent systematic examination, including hysterosalpingography, chromosome analysis of both partners, determination of aPL, including lupus anticoagulant (LA), by diluted activated partial prothrombin time (aPTT), diluted Russell viper venom time (RVVT), and β2-glycoprotein I-dependent anticardiolipin antibody (18), and blood tests for hypothyroidism and diabetes mellitus, before a subsequent pregnancy. Patients with APS, uterine anomalies, and abnormal chromosomes in either partner were excluded from the analysis (19). Patients with a history of preeclampsia or abruptio placentae also were excluded.

Subsequent pregnancies of all patients were followed until February 2013. Among the patients, 225 received no medication and 39 received anticoagulant treatment although there was no evidence. Gestational age was calculated from basal body temperature charts. Ultrasonography was performed once a week from 4 to 8 weeks of gestation. Dilation and curettage was performed on the patients diagnosed as having miscarriage. Part of the villi was cultured, and the cells were harvested after 6–22 days of cultivation to analyze the chromosome. A total of 47 aborted conceptuses could be karyotyped with the use of a standard G-banding technique.

Furthermore, 195 women with at least one child and no history of infertility or miscarriage were examined as control subjects. The control subjects were recruited from Nagoya City University Hospital and Inabe General Hospital from January to April 2012. The earlier study implicated SNP5 (minor allele frequency [MAF] 0.228) as the major susceptibility locus in RPL (17). The minor allele frequency (MAF) of SNP5 was 0.228. Based on a sample size of the 264 patients already obtained, MAF of ~20% in the SNP, and a multiplicative model, 195 control subjects were needed for 80% power and 5% significance.

The allele frequencies of the six SNPs of the *ANXA5* gene were compared between the patients and control subjects. The subsequent pregnancy outcomes were compared between the 264 patients with and without the risk alleles.

This study was conducted with the approval of the Research Ethics Committee of Nagoya City University Graduate School of Medical Sciences and the Ethics Committee of Inabe General Hospital. Each patient provided written consent after being provided with a full explanation about the purpose of the study and the methods to be used.

DNA Analysis

Genomic DNA was extracted from peripheral blood samples with the Midi Blood DNA Extraction kit (Qiagen). A total of six SNPs were analyzed: rs112782763 (−467G>A at promoter; SNP1), rs28717001 (−448A>C at promoter; SNP2), rs28651243 (−422T>C at 5′-untranslated exon 1; SNP3), rs113588187 (−373G>A at 5′-untranslated exon 1; SNP4), rs1050606 (−302T>G; SNP5), and rs11538099 (−1C>T at one base upstream of the initiation codon in exon2; SNP6). All genotyping was carried out using Taqman polymerase chain reaction (PCR) assays (Applied Biosystems) in 96-well arrays that included two blank wells as negative control samples according to the manufacturer's instructions. Taqman Predesigned SNP Genotyping assay and Taqman MGB probes were used. Taqman PCR and genotyping analyses were carried out on the Applied Biosystems 7500 Fast Real-Time PCR System. The reaction mixtures were amplified in 1 μL template DNA (10 ng/μL), 12.5 μL 2× Taqman Universal Master Mix, 0.625 μL 20× primer/probe mix and 10.875 μL double-distilled H_2O in a volume of the mixture of 25 μL. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 58°C for 1 minute. The results were automatically analyzed on the Applied Biosystems 7500 Real-Time PCR System with the use of an allelic discrimination assay program (20).

Statistical Analysis

Departure from the Hardy-Weinberg equilibrium for the six SNPs was tested with the use of an exact test (21). Because previous studies have shown that the mainland Japanese population is genetically similar, we did not examine or perform corrections for the population substructure in our sample (22, 23).

For individual SNP association analyses, univariate logistic regression analyses were performed with dominant and

TABLE 1

Allele frequencies of six *ANXA5* single-nucleotide polymorphisms (SNPs) in patients with recurrent pregnancy loss and control subjects and the odds ratios (ORs) in the codominant and dominant models.

		Case	Control	OR (95% CI)	*P* value	*P* value of max-statistics	HWE	MAF
SNP1	Codominant				.187		0.233	0.110
−467G>A	G/G	206	160	Reference				
rs112782763	G/A	55	30	1.43 (0.87–2.33)				
	A/A	3	5	0.47 (0.11–1.96)				
	Dominant				.287	.443		
	G/G	206	160	Reference				
	G/A, A/A	58	35	1.28 (0.81–2.04)				
SNP2	Codominant				.071		0.086	0.108
−448A>C	A/A	207	162	Reference				
rs28717001	A/C	54	27	1.56 (0.94–2.56)				
	C/C	3	6	0.39 (0.10–1.59)				
	Dominant				.211	.266		
	A/A	207	162	Reference				
	A/C, C/C	57	33	1.35 (0.84–2.17)				
SNP3	Codominant				.086		0.154	0.122
−442T>C	T/T	205	160	Reference				
rs28651243	T/C	56	29	1.51 (0.92–2.50)				
	C/C	3	6	0.39 (0.10–1.59)				
	Dominant				.246	.267		
	T/T	205	160	Reference				
	T/C, C/C	59	35	1.32 (0.01–2.08)				
SNP4	Codominant				.137		0.214	0.107
−373G>A	G/G	207	162	Reference				
rs113588187	G/A	54	28	0.89 (0.92–2.50)				
	A/A	3	5	0.47 (0.11–2.0)				
	Dominant				.211	.381		
	G/G	207	162	Reference				
	G/A, A/A	57	33	1.35 (0.84–2.17)				
SNP5	Codominant				.109		0.099	0.218
−302T>G	T/T	155	132	Reference				
rs1050606	T/G	93	51	1.56 (1.03–2.33)				
	G/G	13	12	1.14 (0.01–2.5)				
	Dominant				.049	.100		
	T/T	155	132	Reference				
	T/G, G/G	109	63	1.47 (1.00–2.17)				
SNP6	Codominant				.044		0.086	0.108
−1C>T	T/T	206	163	Reference				
rs11538099	C/C							
	T/C	55	26	1.67 (1.01–2.78)				
	C/T							
	C/C	3	6	0.40 (0.10–1.61)				
	T/T							
	Dominant				.135	.258		
	T/T	206	163	Reference				
	C/C							
	T/C, C/C	58	32	1.43 (0.89–2.33)				
	C/T, T/T							

Note: CI = confidence interval; HWE = Hardy-Weinberg equilibrium; MAF = minor allele frequency (in both patients and control subjects).

Hayashi. ANXA5 *SNPs in recurrent pregnancy loss. Fertil Steril 2013.*

codominant models using the presence or absence of PRL as the dependent variable. The two inheritance models were tentatively chosen because earlier studies supported a dominant model for these SNPs (16, 17). We estimated the crude odds ratios (ORs) and 95% confidence intervals (CIs) with the use of the two models. However, there remains some uncertainty about the underlying genetic model. Therefore, to examine the right model of inheritance statistically as well as to avoid multiple comparisons by fitting multiple inheritance models, we used the max-statistic, which selects the largest test statistic from the dominant, recessive, and additive models.

To characterize the linkage disequilibrium (LD) pattern, we estimated the r^2 values for all pairs of SNPs. Then, the haplotype frequencies were estimated with the use of the expectation-maximization algorithm. To evaluate the association between the haplotypes and the risk of pregnancy loss, logistic regression models were used. Haplotype analyses were conducted based on the most supported model by the max-statistic in univariate analysis.

In the cohort study, univariate logistic regression analyses were performed to examine the association of subsequent miscarriage with variable SNPs. Then, multivariate logistic analyses were performed to examine the association

Results of maximizing association statistics in the present study and combination with previous data reported by Miyamura et al.

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
Present study						
Dominant	1.132	1.568	1.346	1.568	3.887	2.230
Recessive	1.317	2.177	2.177	1.317	0.002	2.177
Log-additive	0.365	0.400	0.318	0.581	2.408	0.705
max-statistic	1.317	2.177	2.177	1.568	3.887	2.230
P value	.443	.266	.267	.381	.100	.258
Combination with data from Miyamura et al.						
Dominant	4.955	5.826	5.300	6.393	9.943	6.393
Recessive	1.954	2.932	2.932	1.954	1.541	2.932
Log-additive	2.696	2.860	2.565	3.615	9.058	3.177
max-statistic	4.955	5.826	5.300	6.393	9.943	6.393
P value	.056	.035	.046	.025	.003	.025
OR obtained with selection of the dominant model in the combination data						
OR (95% CI), *P* value	1.52 (1.05–2.22), .0029	1.57 (1.09–2.31), .018	1.53 (1.06–2.23), .024	1.62 (1.11–2.39), .013	1.61 (1.20–2.18), .002	1.62 (1.11–2.39), .013

Note: CI = confidence interval; OR = odds ratio; SNP = single-nucleotide polymorphism.

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of subsequent miscarriage with variable SNPs, age, the number of previous miscarriages, and previous live births. The age was quadrisected (21–31, 31–34, 34–37, and 37–45 years), and the number of previous miscarriages (two, three, four, or five to nine) and previous live births (none vs. one or two) were divided so that each group had an almost equal number, and the cells with few numbers were annexed.

Statistical analyses in cross-sectional study were conducted with R software (v. 2.13.0) (24), including the SNPassoc (25) and Haplo.stat (26) packages. Analysis in the cohort study was carried out with SAS (v. 19.0). $P<.05$ was considered to denote statistical significance.

RESULTS

The mean ages (SD) of the patients and control subjects were 33.8 (4.36) and 32.1 (6.91) years, respectively. The mean numbers of previous miscarriage were 2.74 (0.96) and 0, respectively. The mean numbers of previous live births were 0.20 (0.40) and 1.53 (0.72), respectively.

The genotype frequencies for six SNPs were found to be in Hardy-Weinberg equilibrium, suggesting neither sampling bias nor mistyping of genotyping (Table 1). The carrier frequency for the minor alleles tended to be higher in the RPL group for all six SNPs (not significant) when the dominant model was selected (SNP1: G/G vs. G/A-A/A; SNP2: A/A vs. A/C-C/C; SNP3: T/T vs. T/C-C/C; SNP4: G/G vs. G/A-A/A; SNP5: T/T vs. T/G-G/G; and SNP6: C/C vs. C/T-T/T), among which the highest significance level was obtained for SNP5 ($P=.049$; Table 1). When we selected the codominant model (SNP1: G/G vs. G/A vs. A/A; SNP2: A/A vs. A/C vs. C/C; SNP3: T/T vs. T/C vs. C/C; SNP4: G/G vs. G/A vs. A/A; SNP5: T/T vs. T/G vs. G/G; and SNP6: C/C vs. C/T vs. T/T), the highest significance level was obtained for SNP6 ($P=.044$).

Then we merged our data with the previously published Japanese data (17). Based on the results of the max-statistics, the dominant model was adopted for all six polymorphisms (Table 2). The combined data suggested that the dominant model should be selected, and statistically significant correlations were obtained for all six SNPs, with the highest level of significance for SNP5 ($P=.003$). However, the ORs were <2.0 for all six SNPs.

We next performed LD analysis among the six *ANXA5* SNPs (Fig. 1). LD evaluation revealed the existence of significant correlations among SNPs 1, 2, 3, 4, and 6. All except SNP5 exhibited a strong LD (>0.95), as reported previously (16).

Haplotype analysis indicated the presence of three major haplotypes. G-A-T-G-T-C (patient vs. control: 75.9% vs. 80.3%) and G-A-T-G-G-C (12.1% vs. 8.7%) were the major haplotypes and accounted for >85% of the subjects. The first four (G-A-T-G) SNPs constituted the N haplotype described previously (15). The haplotypes comprising all six minor alleles (A-C-C-A-G-T) was found to be the third most common (11.4% vs. 9.2%). The first four SNPs (A-C-C-A) constituted the M2 haplotype. All other haplotypes accounted for <1%. We subsequently performed a case-control study for SNPs 1–6 and for these three haplotypes. When we selected the dominant model, there was no significant difference of the