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Reduced Stathmin-1 Expression in Natural Killer Cells Associated with Spontaneous Abortion

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Female CBA/J mice impregnated by male DBA/2J mice (CBA/J×DBA/2J matings) are prone to spontaneous abortion, although the reason for this is unclear. In this study, the stathmin-1 expression pattern was evaluated in uterine natural killer (uNK) cells purified from CBA/J×DBA/2J matings. Results were compared with those in a CBA/J×BALB/c control group that yields successful pregnancies. The mean ± SD percentage of stathmin-1⁺ cells in the CD49b⁺ uNK cell population was lower in CBA/J×DBA/2J mice (0.7% ± 0.4%) than in control CBA/J×BALB/c mice (4.9% ± 1.5%, *P* < 0.01) using flow cytometry, and the intracellular stathmin-1 level in uNK cells was lower in CBA/J×DBA/2J mice than in control mice using Western blot analysis. Co-localization of lectin from *Dolichos biflorus* agglutinin (DBA-lectin) and stathmin-1 was confirmed using multivision immunohistochemical analysis. The frequency of stathmin-1⁺DBA-lectin⁺ cells was lower in CBA/J×DBA/2J mice than in CBA/J×BALB/c mice. A similar trend in the frequency of stathmin-1⁺CD56⁺ cells was seen in patients with unexplained spontaneous abortion compared with normal early pregnancy. A neutralizing antibody

against stathmin-1 further increased the percentage of embryo loss in CBA/J×DBA/2J matings. These results provide evidence that stathmin-1 expression in uNK cells at the maternal-fetal interface may help modulate uNK cell function and may be beneficial for a successful pregnancy. (*Am J Pathol* 2011, 178:506–514; DOI: 10.1016/j.ajpath.2010.10.005)

Stathmin-1 is a small (19-kDa) regulatory phosphoprotein that integrates diverse intracellular signaling pathways. It is highly conserved among vertebrates and is associated with tubulin binding and microtubule destabilization.^{1,2} Stathmin-1 has a complex phosphorylation pattern in response to various extracellular signals, in particular growth and differentiation factors.³ Moreover, stathmin-1 phosphorylation varies during the cell cycle.⁴ It has thus been thought that stathmin-1 can act as a relay integrating the activation of diverse intracellular signaling pathways and mediating the control of cell proliferation, differentiation, and other functions.⁵

Stathmin-1 protein and mRNA were previously shown to be expressed in the pregnant uterus and decidualizing endometrial stromal cells in human and murine models.^{6–8} Furthermore, stathmin-1 is up-regulated in rodent uteri at the site of embryo implantation and is highly

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expressed in the decidual zone during the decidualization process.^{7,8} These results suggest that stathmin-1 may participate in the modulation of embryo implantation and decidualization.

Female CBA/J mice impregnated by male DBA/2J mice (CBA/J×DBA/2J matings) are prone to abortion, in contrast to the major histocompatibility complex-identical CBA/J×BALB/c matings, which are resistant to abortion.⁹ The underlying mechanisms for these observations are unclear. Clark and colleagues⁹ suggested that endothelium is the primary effector cell population, and this was supported by a recent work using CBA/J×DBA/2J matings.¹⁰ Notably, inhibition of natural killer (NK) cells using anti-asialo GM1 antiserum significantly decreased the resorption rate of embryos in CBA/J×DBA/2J matings.⁹ In the present study, uterine NK (uNK) cells were purified from CBA/J×DBA/2J and CBA/J×BALB/c allogeneic pregnant models using magnetic affinity cell sorting (MACS). The percentage of stathmin-1⁺ cells in the uNK cell population was determined using flow cytometry, and the stathmin-1 protein expression level in uNK cells was determined using two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), and Western blot analysis. Multivision immunohistochemical analysis (IHC) was used to examine the distribution patterns of stathmin-1⁺ cells in the uteri of pregnant female mice and in first-trimester human decidual tissue. In addition, inhibition of stathmin-1 was performed in CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J mice. From these data, the possible role of stathmin-1 in allogeneic pregnancy tolerance was investigated.

Materials and Methods

Pregnant Models of CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J Matings

Female CBA/J mice and male CBA/J, DBA/2J, and BALB/c mice (8 to 12 weeks old) were purchased from the Model Animal Center of Nanjing University (Nanjing, China) and were housed under specific pathogen-free conditions. Pregnant models of CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J matings were established by co-caging female CBA/J mice with DBA/2J, BALB/c, and CBA/J males, respectively. Detection of a vaginal plug was chosen to indicate day 0.5 of gestation (E0.5).^{11,12} Embryonic day E12.5 was chosen as the gestational time to collect uNK cells because the uNK cells are at peak density on day E10 and have not yet begun to decrease in density through apoptosis (which begins on day E13 or E14).¹³ Furthermore, we expected that it would be easier to distinguish healthy embryos from resorbing ones on day E12.5 than at an earlier time point. All animal procedures followed the national animal care guidelines, and associated data were approved for publication by the institutional review board of Shanghai Ji-aotong University.

Purification of uNK Cells

Cell purification was performed by means of MACS.^{11,12} Briefly, hysterolaparotomy was performed on day E12.5 to collect embryo-depleted placentas from CBA/J×DBA/2J and CBA/J×BALB/c matings. The uterine horns were opened longitudinally, and the fetoplacental unit was separated easily from the uterine implantation sites. The whole placental and decidual unit was separated individually from the respective embryo. The pooled placentas and decidua basalis (ie, decidual tissue in implantation sites) were collected into a dish and carefully cut into small pieces, collected in 0.9% NaCl solution, and subsequently filtered through a nylon mesh (50- μ m pore size) to obtain a single cell suspension. Mononuclear cells were obtained by centrifuging of the single cell suspension using a Ficoll-Hipaque density column. Any red blood cells that contaminated the single cell suspension were eliminated by incubation with red blood cell lysis buffer (eBioscience Inc., San Diego, CA) two times at 37°C. Subsequently, NK cells were isolated using magnetic bead-conjugated antimouse CD49b monoclonal antibody, and CD49b⁺ cells were purified by means of Mini MACS columns (Miltenyi Biotec Inc., Auburn, CA),^{11,12} where CD49b was used as a common marker for murine NK cells.¹⁴ The purity of the MACS-isolated NK cells routinely exceeded 95% as determined using flow cytometry.^{12,15}

Flow Cytometry

Uterine NK cells were stained with phosphatidylethanolamine (PE)-conjugated antimouse CD49b (BioLegend, San Diego, CA) and rabbit anti-stathmin-1 (catalog number ab52906; Abcam, Cambridge, England) antibodies, followed by fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (Molecular Probes Inc., Eugene, OR). The percentage of stathmin-1⁺ cells in the CD49b⁺ NK cell population was determined by using a flow cytometer (FACS Aria; BD Biosciences, Franklin Lakes, NJ).¹¹ Cells were stained with PE-conjugated antimouse CD49b and FITC-conjugated antimouse CD122 antibodies (both from BioLegend) to determine the percentage of CD122⁺ cells in the CD49b⁺ population. Isotype controls were established by using isotype control antibodies (BioLegend) to exclude false-positive cells. All the experiments were independently performed six times.^{16–18}

Preparation of uNK Cell Lysates

Uterine NK cells were suspended in a modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 0.66 μ g/ml of aprotinin, 0.5 μ g/ml of leupeptin, 1 μ g/ml of pepstatin, 1 mmol/L Na₃VO₄, and 1 mmol/L NaF] and were sonicated three times for 5 seconds each. The cell lysates were centrifuged at 14,000 $\times g$ for 15 minutes at 4°C. The supernatants were collected, and their protein concen-

trations were measured by using the Bradford assay (Bio-Rad Laboratories, Hercules, CA).^{19,20}

Two-Dimensional Gel Electrophoresis

The 2-DE was performed according to the manufacturer's instructions. Samples were loaded onto linear immobilized pH gradient (IPG) strips (IPGstrip, pH 4–7 L, 180 × 3 × 0.5 mm; Amersham Biosciences, Piscataway, NJ). Briefly, 1-mg protein samples were diluted to 350 μ L with a rehydration solution [7 mol/L urea, 2 mol/L thiourea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1 propanesulfonate, 18 mmol/L dithiothreitol, 0.5% (v/v) pH 4–7 IPG buffer, and trace bromophenol blue] and were applied to the IPG strips with 14 hours of rehydration at 30 V. The proteins were successively focused for 1 hour at 500 V, 1 hour at 1000 V, and 5 hours at 8000 V for a total of 41,920 V hours on an IPGphor (Amersham Biosciences). The focused IPG strips were equilibrated for 15 minutes in solution (6 mol/L urea, 2% SDS, 30% glycerol, 50 mmol/L Tris-HCl, pH 8.8, and 1% dithiothreitol) and then for an additional 15 minutes in the same solution containing 2.5% iodoacetamide instead of dithiothreitol. After equilibration, SDS–polyacrylamide gel electrophoresis was performed at 10°C on 10% SDS slab gels using the Ettan DALT II system (Amersham Biosciences) with the IPG strips sealed on the top of the gels with 0.5% agarose. An SDS–polyacrylamide gel electrophoresis was performed at a constant power of 2W/gel for 30 minutes and then switched to 12 W/gel until the bromophenol blue marker reached the bottom of the gel. Finally, the blue silver staining method (a modified Neuhoff's colloidal Coomassie Blue G-250 stain) was used to visualize the protein spots in the 2-DE gels.^{17,20}

2-DE Image Data Analysis

Stained 2-DE gels were scanned using LabScan software and ImageScanner (Amersham Biosciences) at a resolution of 300 dpi. Spot-intensity calibration, spot detection, matching, 1-D calibration, and establishment of an average gel were performed using the PDQuest System (Bio-Rad Laboratories). The theoretical molecular weight and pI value of the identified protein spots were calculated according to algorithms included in the PDQuest analysis software package. Significant differences in the protein expression levels were determined using the *t*-test, with significance defined at $P < 0.05$.^{17,20}

Preparation of Protein Spots

Protein spots were excised from the preparative gels and were placed into a 96-well microtiter plate. Proteins were digested in gel as previously described.^{17,20} The gel spots were destained using destaining solution [200 mmol/L NH_4HCO_3 and 100% acetonitrile (1:1)] for 20 minutes at room temperature. Gel spots were washed twice with deionized water, shrunk by dehydration in acetonitrile solution, and dried in a vacuum centrifuge. Samples were then swollen in a digestion buffer (20 mmol/L ammonium bicarbonate and 12.5 ng/ μ L of trypsin) (Sig-

ma-Aldrich, St. Louis, MO) for 30 minutes at 4°C. The gels were then digested for 12 hours at 37°C. Tryptic peptides were extracted twice from the gel slices by sonication for 15 minutes in a 0.1% trifluoroacetic acid/50% acetonitrile solution. The supernatants were collected and dried to a pellet in a high-purity nitrogen flow. Peptides were eluted with 0.7 μ L of α -cyano-4-hydroxycinnamic acid matrix solution and were loaded onto a stainless steel target with 192 wells (Applied Biosystems, Framingham, MA).^{17,20}

Mass Spectrometry

Samples were air-dried and then were analyzed by using the Voyager System 4700 matrix-assisted laser desorption/ionization–time of flight–time of flight mass spectrometer (Applied Biosystems).^{17,20}

Protein Identification

Known contaminating peaks (eg, keratin and autolysis peaks) were removed before the database search. Spectra were processed and analyzed using a GPS Explorer (Applied Biosystems). Mascot software (Matrix Science, London, England) was used to search for peptide mass fingerprints and MS/MS data in the NCBI nr database. Protein scores by Mascot search analysis that were >63 were considered significant ($P < 0.05$).^{17,20}

Western Blotting Analysis

Tissue aliquots were homogenized to powder using liquid nitrogen and then were dissolved in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-Cl, pH 8.0, 0.1% Nonidet P-40 (Caledon Laboratories Ltd., Georgetown, Ontario, Canada), 1 mmol/L phenylmethylsulfonyl fluoride, 25 μ g/ml of aprotinin, and 25 μ g/ml of leupeptin], vortexed, and incubated at room temperature for 2 hours. The mixture was centrifuged at 20,644 × *g* for 30 minutes at 4°C, and the supernatant was used as the total protein solution. The lysate concentration was assayed using the Bradford assay.

Western blotting analysis was performed as previously described.^{17,20} Briefly, 100 μ g of total protein was separated on a 12% SDS–polyacrylamide gel electrophoresis gel before being transferred onto a nitrocellulose membrane. After blocking with 5% milk in Tris-buffered saline/0.2% Tween 20 for 1 hour at room temperature, the membrane was incubated with rabbit antimouse stathmin-1 antibody (1:100 dilution) (Abcam) for 1 hour at room temperature, followed by incubation with horseradish peroxidase–conjugated goat antirabbit IgG secondary antibody (1:10,000 dilution; Amersham Biosciences) for 1 hour at room temperature. Detection of NADPH was used as a loading control. Reactions were visualized using an enhanced chemiluminescence detection system (ECL; Amersham Biosciences). Signals on the blots were visualized using autoradiography.

Multivision IHC of Placental Tissue in Murine Models

Placentas together with decidua basalis harvested on day E10.5 from CBA/J×DBA/2J and CBA/J×BALB/c matings (none of which were analyzed by means of 2-DE) were used to measure the distribution of stathmin-1 protein in lectin from *Dolichos biflorus* agglutinin–positive (DBA-lectin⁺) cells using a multivision IHC procedure. Paraffin-embedded tissue blocks were cut into 4- μ m-thick sections, which were then deparaffinized in xylene and rehydrated in graded alcohol concentrations. Nonspecific binding was further blocked by preincubation with blocking solution for 5 minutes, followed by incubation for 1 hour at 4°C with rabbit antimouse stathmin-1 (1:200 dilution) (Cell Signaling Technology Inc., Beverly, MA). Meanwhile, FITC-conjugated DBA-lectin (1:200 dilution) (Sigma-Aldrich) was added onto the section in the dark for 1 hour. The sections were then washed three times with PBS for 5 minutes each and incubated with PE-conjugated antirabbit IgG (1:200 dilution) (Alpha Diagnostic International, San Antonio, TX) for 30 minutes at room temperature in the dark. Then, 4',6-diamidino-2-phenylindole (Invitrogen, San Diego, CA) was used to stain nuclei for 10 minutes in the dark. Negative controls were established using rabbit Ig of the isotype identical to the rabbit antimouse primary antibody in place of the specific primary antibody (Cell Signaling Technology Inc.).²¹

Multivision IHC of Human Decidual Tissue

First-trimester human decidual tissue was obtained from five normal pregnancies (free of spontaneous abortion history; mean $\pm$ SD age, 27.5 $\pm$ 2.2 years; mean $\pm$ SD gestational age at sampling, 8.2 $\pm$ 1.1 weeks, terminated for nonmedical reasons) and five miscarriages [maternal history of more than three unexplained recurrent spontaneous abortions (RSAs); mean $\pm$ SD age, 32.4 $\pm$ 3.9 years; mean $\pm$ SD gestational age at sampling, 8.5 $\pm$ 2.8 weeks], which were classified as unexplained after the exclusion of maternal anatomical or hormonal abnormalities and paternal or maternal chromosomal abnormalities. All the samples were obtained from Renji Hospital, Shanghai Jiaotong University, with written informed consent from the patients and permission from the research ethics committee of Shanghai Jiaotong University.

To confirm the existence and define the distribution pattern of CD56⁺stathmin-1[−] and CD56⁺stathmin-1⁺ cells in human decidual tissue, paraffin sections were stained with rabbit antihuman stathmin-1 (Abcam) and mouse antihuman CD56 (Lab Vision/NeoMarkers, Fremont, CA) monoclonal antibodies, followed by staining with multivision antirabbit/horseradish peroxidase (horseradish peroxidase/diaminobenzidine) plus antimouse/alkaline phosphatase polymers (Biolab Science, Beijing, China), according to the manufacturers' instructions. Using this multivision polymer detection system, stathmin-1⁺ cells were stained brown, CD56⁺ cells were stained red, and double-positive cells were double colored. Nuclei were lightly stained with hematoxylin.

Inhibition of Stathmin-1 in Vivo

Inhibition of stathmin-1 was performed in CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J matings by i.p. injection of anti-stathmin-1 antibody (GenScript USA Inc., Piscataway, NJ) on days E4.5, E5.5, and E6.5 (20 μ g in 0.2 ml of PBS) once a day. Mice injected with the same volume of rabbit IgG isotype control antibody were used as controls for each group. The percentage of embryo resorption was detected on day E12.5 by using the method described previously herein (n = 8 per group).

Statistical Analysis

Flow cytometry data were analyzed by using Quad statistics.¹⁶ The resorption rate was compared using the χ^2 test, and the cell percentage was compared using the independent-samples *t*-test. Cell percentage results are presented as mean $\pm$ SD.^{17,18} Significance was defined at P < 0.05.

Results

The Percentage of Embryo Loss Is Increased in CBA/J×DBA/2J Matings

The percentage of spontaneously resorbed embryos on day E12.5 was 22.6% (35 of 155; n = 16) in CBA/J×DBA/2J matings, 7.6% (13 of 170; n = 18) in CBA/J×BALB/c matings, and 7.2% (8 of 111; n = 12) in CBA/J×CBA/J matings. There was no significant difference between CBA/J×BALB/c and CBA/J×CBA/J matings in the percentage of embryo loss, whereas the percentage of embryo resorption in CBA/J×DBA/2J matings was significantly higher than that in CBA/J×BALB/c and CBA/J×CBA/J matings (P < 0.01 for both). The increased resorption rate of CBA/J×DBA/2J matings supports the hypothesis that these mice are prone to spontaneous embryo loss.

Flow Cytometric Analysis of MACS-Purified CD49b⁺ Cells

The mean $\pm$ SD percentage of stathmin-1⁺ cells in the CD49b⁺ NK cell population was approximately six-fold higher in CBA/J×BALB/c matings (5.2% $\pm$ 1.5%) than in CBA/J×DBA/2J matings (0.9% $\pm$ 0.4%) (P < 0.01), as indicated by flow cytometry (Figure 1).

Two-color flow cytometry revealed that most CD49b⁺ cells also expressed CD122 molecules in both matings. The mean $\pm$ SD percentage of CD122⁺CD49b⁺ cells in the CD49b⁺ population was 74.9% $\pm$ 11.4% in CBA/J×DBA/2J matings, which was not significantly different from that in CBA/J×BALB/c matings (65.6% $\pm$ 8.8%).

Reduced Stathmin-1 Production in uNK Cells in CBA/J×DBA/2J Matings in 2-DE

Proteins from two sets of pooled (12 pregnant mice per group) uNK cell lysates isolated from CBA/J×DBA/2J

Figure 1. Flow cytometric analysis of stathmin-1⁺ and CD122⁺ cells in uNK cells. **A–H:** Representative results derived from cells purified using microbead-conjugated anti-CD49b and a Mini MACS. **A, B, E, F:** Isotype controls established using cells stained with FITC- and PE-conjugated isotype antibodies. **C, D:** Analysis of stathmin-1⁺ cells in the CD49b⁺ cell population. The cell percentage is indicated. **G, H:** Analysis of CD122⁺ cells in the CD49b⁺ cell population. **I, J:** Data summary of flow cytometry. Experiments were independently repeated six times in each group. The mean $\pm$ SD percentage of stathmin-1⁺CD49b⁺ cells was lower in CBA/J×DBA/2J mice than in CBA/J×BALB/c mice ($0.9\% \pm 0.4\%$ versus $5.2\% \pm 1.5\%$, $P < 0.01$). A strong CD122 signal was detected in both mating combinations. The mean $\pm$ SD percentage of CD122⁺CD49b⁺ cells in the CD49b⁺ population was $74.9\% \pm 11.4\%$ in CBA/J×DBA/2J mice and $65.6\% \pm 8.8\%$ in CBA/J×BALB/c mice, suggesting that most of the MACS-purified CD49b⁺ cells also express CD122. Error bars represent SD. ****** $P < 0.01$.

matings and age-matched CBA/J×BALB/c matings on day E12.5 were resolved using 2-DE. These experiments were repeated four times under identical experimental conditions and parameters to confirm reproducibility. Well-resolved and reproducible Coomassie Brilliant Blue-stained 2-DE maps from CBA/J×DBA/2J and CBA/J×BALB/c matings were obtained. The intensity of the stathmin-1 protein spot derived from CBA/J×BALB/c matings was approximately four-fold higher than that in CBA/J×DBA/2J matings (mean $\pm$ SD, 4.1 ± 0.9 -fold; $P < 0.01$) (Figure 2).

Identification and Quantification of Stathmin-1 Protein Using Matrix-Assisted Laser Desorption/Ionization–Time of Flight–MS

In the 2-DE analysis described previously herein, differentially expressed protein spots were excised from Coomassie Brilliant Blue-stained gels and were subjected to in-gel digestion with trypsin. An aliquot of the supernatant containing tryptic peptides was analyzed by means of matrix-assisted laser desorption/ionization–time of flight–MS/MS, and the Mascot search program software package was used to identify the analyzed protein spots. Proteins receiving a significant score >63 ($P < 0.05$) in the Mascot database were ranked as the best hits. Stathmin-1 was one protein that was differentially expressed between the two sets of samples. The protein spot shown in Figure 2 was analyzed using MS and was identified as stathmin-1 (Figure 3). Stathmin-1 received a protein score of 280, a molecular weight of 17,264 Da, a pI of 5.76, and accession number gi 9789995.

The differential expression of stathmin-1 was further confirmed by immunoblots using a stathmin-1-specific antibody. The intensity of the stathmin-1 spot in CBA/J×BALB/c matings was a mean $\pm$ SD 6.9 ± 2.2 -fold higher than that in CBA/J×DBA/2J matings ($*P < 0.01$) (Figure 4).

Figure 2. Stathmin-1 expression determined using 2-DE. **A–D:** Map of uterine lymphocyte lysates from CBA/J×BALB/c mice (**A** and **C**) or CBA/J×DBA/2J mice (**B** and **D**). **C** and **D** indicate the original magnification of the stathmin-1 protein spot from CBA/J×BALB/c and CBA/J×DBA/2J mice, respectively. The density of the stathmin-1 protein spot from CBA/J×BALB/c mice was higher than that from CBA/J×DBA/2J mice as observed by the naked eye. **E:** Mean density as determined using Image-Pro Plus 6.0. The mean density of stathmin-1 spots in CBA/J×BALB/c mice was a mean $\pm$ SD 4.1 ± 0.9 -fold higher than that in CBA/J×DBA/2J mice (****** $P < 0.01$). Experiments were independently performed four times. Error bars represent SD.

Figure 3. Mass spectrometry of the stathmin-1 spot. MS/MS signals were derived from the parent ion, for which the amino acid sequence, QIT-VNDLPVGR, was deduced based upon these ions in tandem MS spectrum. MS/MS spectrum of stathmin-1 is shown. Peptide was identified by matrix-assisted laser desorption/ionization time of flight/time of flight MS/MS and matched with residues 140–150 of stathmin-1. Protein sequence of stathmin-1 is shown, and matched MS/MS fragmentation is underlined. The peptide mass fingerprinting and the MS/MS maps of stathmin-1 were combined to confirm the MS results.

Disparate Abundance of Stathmin-1⁺DBA-Lectin⁺ Cells between CBA/J×BALB/c and CBA/J×DBA/2J Matings

As shown in Figure 5, the presence of stathmin-1⁺DBA-lectin⁺ cells was confirmed in CBA/J×BALB/c and CBA/J×DBA/2J matings using multivision IHC. The DBA-lectin⁺ cells were stained green, stathmin-1⁺ cells were stained red, and double-positive cells were yellow in the merged images. Nuclei were stained blue by 4',6-diamidino-2-phenylindole.

However, the frequency of stathmin-1⁺DBA-lectin⁺ cells was significantly higher in CBA/J×BALB/c matings, whereas DBA-lectin⁺stathmin-1⁺ cells were rare in CBA/J×DBA/2J matings. As indicated in Figure 5, A–D, DBA-lectin⁺ cells (green) are more dominant than stathmin-1⁺ cells in both mating combinations. However, red (stathmin-1) is robust in CBA/J×BALB/c matings but either absent or faint in CBA/J×DBA/2J matings. In the merged images, the relative number of stathmin-1⁺DBA-lectin⁺ cells (yellow) is higher in CBA/J×BALB/c matings (Figure 5, A and B) than in CBA/J×DBA/2J matings (Figure 5, C and D).

The murine cells positive for DBA-lectin were scattered intensively in decidual basalis tissue and in the mesometrial lymphoid aggregate of pregnancy in both matings, whereas no DBA-lectin⁺ cells were observed in placenta tissue (region P) (Figure 5, E and F).²²

The mean density of stathmin-1⁺DBA-lectin⁺ cells was determined using Image-Pro Plus 6.0 (Media Cybernetics Inc., Bethesda, MD). The mean ± SD density of the double-positive cells was significantly higher in CBA/J×BALB/c matings than in CBA/J×DBA/2J matings (9.6 ± 5.5 versus 1.0 ± 0.9; *P* < 0.01) (Figure 5G).

The Distribution Pattern of Stathmin-1⁺ Cells in the Human CD56⁺ Cell Population

The distribution pattern of stathmin-1[−]CD56⁺ and stathmin-1⁺CD56⁺ cells was evaluated by means of double-vision IHC. Stathmin-1⁺CD56⁺ cells were observed in human decidua from RSA patients (Figure 6, A–D) and those with normal early pregnancy (Figure 6, E–H) but were more frequently detected in the latter. Stathmin-1[−]CD56⁺ and stathmin-1⁺CD56⁺ cells were mainly detected in tissues near blood vessels, whereas stathmin-1[−]CD56⁺ cells were seldom found in tissues where there were almost no blood vessels. The mean density of stathmin-1⁺CD56⁺ cells was determined using Image-Pro Plus 6.0. The mean ± SD density of the double-positive cells was significantly higher in normal early pregnancy than in RSA patients (13.6 ± 7.2 versus 1.0 ± 0.7; *P* < 0.01) (Figure 6I).

Effects of Anti-Stathmin-1 Antibody Treatment on Embryo Loss

The mean ± SD percentage of embryo resorption was higher in CBA/J×DBA/2J matings when stathmin-1 protein was inhibited using a neutralizing antibody (with inhibition: 42.1% ± 21.2%, 32 of 76; control: 21.8% ± 12.4%, 17 of 78; *P* < 0.05) (Figure 7). The change in the percentage of embryos lost in the CBA/J×BALB/c and CBA/J×CBA/J matings treated with the stathmin-1 neutralizing antibody did not reach statistical significance (CBA/J×BALB/c, with inhibition: 14.1% ± 10.1%, 11 of 78; CBA/J×BALB/c, control: 8.5% ± 5.4%, 6 of 71; CBA/J×CBA/J, with inhibition: 9.9% ± 6.5%, 8 of 75; CBA/J×CBA/J, control: 7.3% ± 6.5%; 6 of 79) (Figure 7).

Discussion

Stathmin is reportedly expressed in the glandular epithelium and stromal cells of human endometrial tissue by cytotrophoblasts and extravillous trophoblasts but not by syncytiotrophoblasts.⁶ When stromal cells isolated from normal endometrial tissues were previously cultured and stimulated to decidualize by progesterone plus estrogen or cAMP, their total and phosphorylated stathmin levels decreased.

Figure 4. Western blot analysis of stathmin-1 expression in CBA/J×DBA/2J and CBA/J×BALB/c matings. **A:** Stathmin-1 expression is significantly down-regulated in CBA/J×DBA/2J mice compared with CBA/J×BALB/c mice. NADPH was used as an internal loading control. **B:** Histogram showing the relative expression level of stathmin-1 protein in CBA/J×BALB/c and CBA/J×DBA/2J mice as determined using Image-Pro Plus 6.0. The stathmin-1 protein level is a mean ± SD 6.9 ± 2.2-fold higher in CBA/J×BALB/c mice than in CBA/J×DBA/2J mice as determined using densitometric analysis (***P* < 0.01). Experiments were independently repeated four times for each group. Error bars represent SD.

Figure 5. The distribution pattern of stathmin-1⁺DBA-lectin⁺ cells in murine tissue. In multi-vision IHC, uNK cells were stained green with FITC-conjugated DBA-lectin, stathmin-1⁺ cells were indirectly stained red with rabbit anti-stathmin-1 and PE-conjugated antirabbit IgG, and nuclei were stained blue with DAPI. In the merged images, stathmin-1⁺DBA-lectin⁺ cells appear yellow or orange (arrows) and stathmin-1⁺DBA-lectin⁺ cells are green (arrow-heads). **A** and **B**: From CBA/JxDBA/2J mice. **C** and **D**: From CBA/JxDBA/2J mice. **A** and **B** as well as **C** and **D** are presented to show reproducibility. Scale bar = 10 μ m. The DBA-lectin⁺ cells were dominant in both mating combinations compared with stathmin-1⁺ cells and other indicated cell subsets. In the merged images from CBA/JxDBA/2J matings, red (stathmin-1) is either absent or faint. In contrast, red (stathmin-1) is robust in the images from CBA/JxDBA/2J matings and readily yield yellow and is visible in the presence of green (DBA-lectin). **E**: From CBA/JxDBA/2J mice. **F**: From CBA/JxDBA/2J mice. Original magnification, $\times 40$ (**E** and **F**). P, placenta; D, decidua basalis; M, mesometrial lymphoid aggregate of pregnancy. The DBA-lectin⁺ cells were scattered intensively in regions D and M in both matings, but no positive cells were found in region P. **G**: The mean density of stathmin-1⁺DBA-lectin⁺ cells as determined using Image-Pro Plus 6.0. Error bars represent SD. ** $P < 0.01$.

Stathmin silencing in primary stromal cells using small interfering RNA before the cells are exposed to decidualizing agents also markedly suppresses decidualization, suggesting that stathmin may play a key role in decidualization.⁶ Stathmin overexpression favors microtubule destabilization, whereas decreased stathmin expression favors elongated, bundled microtubules and an increased ratio of polymerized to soluble tubulin.²³ Immunohistochemical analyses using a rat model previously revealed that stathmin-1 is exclusively localized in decidual cells, especially in the primary decidual zone surrounding the embryo with markedly more intense staining on day E9.5 than on day E7.5. On day E14.5, when the endometrial stromal cells have completely differentiated into decidual cells, the staining of decidual cells is faint.⁷ An experiment in the delayed implantation pregnant rat model revealed low uterine stathmin expression that was increased after implantation, which was induced by 17 β -estradiol administration to progesterone-primed animals. Furthermore, decidualization in the pseudopregnant rat, induced by intrauterine oil infusion, stimulates stathmin expression. Stathmin expression clearly increases in the uterus when stimulated by embryo implantation and decidualization and is believed to play a role in the early stages of pregnancy.⁸

Herein, we demonstrated by using two-color flow cytometry that stathmin-1 is expressed intensively in uNK cells isolated from mouse models. The uNK cells were previously

purified by means of MACS and were confirmed to be CD3⁺CD49b⁺ cells.¹¹ In multi-vision IHC using decidual samples from humans and CD56 as a pan marker for human NK cells, the distribution pattern of stathmin-1⁺CD56⁺ cells was consistent with the reported roles of NK cells in the pregnant uterus. Under physiologic conditions, NK cells are the dominant cell population up to mid-gestation in the pregnant uterus and are believed to participate in blood vessel remodeling.^{24–26} The origin of these NK cells is unclear. A previous study demonstrated that NK cells from extrauterine tissue migrate into the pregnant uterus.²⁶ Thus, we conclude that a fraction of the stathmin-1⁺ cells are actually uNK cells, which immigrate into the pregnant uterus at the early stages of pregnancy and are involved in the modulation of implantation and decidualization.

The percentage of stathmin-1⁺ cells in the uNK cell population was significantly higher in CBA/JxDBA/2J matings than in abortion-prone CBA/JxDBA/2J matings. In addition, the intensity of stathmin-1 expression was stronger in CBA/JxDBA/2J matings than in CBA/JxDBA/2J matings, as indicated by 2-DE and Western blot analysis. This differential stathmin-1 expression correlated with the difference in the embryo loss rate, which was lower in CBA/JxDBA/2J matings than in CBA/JxDBA/2J matings. Thus, the reduced stathmin-1 production in uNK cells correlates with the increased failure of healthy embryos by CBA/JxDBA/2J matings. Furthermore, stathmin-1 inhibition with a neutralizing

antibody increased the percentage of embryo loss in CBA/J×DBA/2J matings, but no such trend was observed in CBA/J×BALB/c, CBA/J×CBA/J, or syngeneic CBA/J×CBA/J matings. These results suggest that stathmin-1 may be a key regulator in the maintenance of allogeneic pregnancy tolerance, at least in CBA/J×DBA/2J matings. In CBA/J×BALB/c matings, the increase in embryo resorption after stathmin-1 inhibition did not reach statistical significance. Although the reason for this is unclear, it may be explained by the difference in the uNK cell functional status between CBA/J×DBA/2J and CBA/J×BALB/c matings, indicating the presence of a fragile system of stathmin-1-mediated modulation in CBA/J×DBA/2J matings.

In multivision IHC, colocalization of stathmin-1 and DBA-lectin was confirmed in murine decidual tissue from both mating combinations. However, the frequency of

Figure 7. Effect of anti-stathmin-1 antibody treatment on the embryo resorption rate. No inhibition, multiple injections with isotype control antibody; inhibition, multiple injections with neutralizing antibody. In the no-inhibition CBA/J×DBA/2J group, the percentage of embryo loss was significantly higher than that in the no-inhibition CBA/J×BALB/c and CBA/J×CBA/J groups, confirming that CBA/J×DBA/2J mice are prone to abortion. The percentage of embryo loss was increased after stathmin-1 inhibition in CBA/J×DBA/2J mice (* $P < 0.05$, ** $P < 0.01$), but the trend of embryo loss increase did not reach statistical significance in CBA/J×BALB/c or CBA/J×CBA/J mice. $n = 8$ per group. Error bars represent SD.

Figure 6. The distribution pattern of stathmin-1+CD56+ cells in human decidua. The distribution pattern of stathmin-1+CD56+ cells was examined by means of double-staining IHC in decidua from RSA patients (A–D) and subjects undergoing healthy elective pregnancy termination (E–H). Single CD56+ cells are stained red (arrowheads), stathmin-1+CD56+ cells are stained brown (arrows), and nuclei are stained blue with hematoxylin. Original magnification is marked. B, D, F, and H are local magnifications of the regions enclosed with boxes in A, C, E, and G, respectively. I: Mean density of stathmin-1+CD56+ cells as determined using Image-Pro Plus 6.0. Error bars represent SD. ** $P < 0.01$.

stathmin-1+DBA-lectin+ cells was significantly higher in CBA/J×BALB/c mice than in CBA/J×DBA/2J mice (Figure 5). Stathmin-1+DBA-lectin+ cells were found mainly scattered in the decidua basalis and mesometrial lymphoid aggregate of pregnancy in the murine pregnant uteri. Because the decidua basalis and mesometrial lymphoid aggregate of pregnancy, to some extent, represent the maternal-fetal interface and because more immunopotent cells can infiltrate into these tissues than into other parts of murine placental and decidual tissues, these results suggest that stathmin-1+DBA-lectin+ NK cells may be important in the modulation of maternal-fetal cross talk.^{25,26} Similar results were obtained in human decidual tissue. Using CD56 as a pan-NK cell marker for human uNK cells, we found that the frequency of stathmin-1+CD56+ cells was significantly higher in decidual tissues from normal early pregnancy than in those from spontaneous abortion patients (Figure 6). This suggests that some cases of unexplained spontaneous miscarriage may be attributable to reduced function of uNK cells, including reduced production of stathmin-1 protein in uNK cells. In addition, stathmin-1+CD56+ cells were found mainly scattered in tissues near blood vessels, suggesting that they may participate in establishment, remodeling, or other functions of the blood vessel system. Stathmin-1+CD56+ cells were less frequently detected in RSA samples, consistent with the results obtained using mouse two-color flow cytometry. Taken together, the results of the present study suggest that a fraction of uNK cells express stathmin-1 molecules and that insufficient stathmin-1 expression in uNK cells may be related to increased embryo loss in abortion-prone mice and some patients with RSA.

In addition to murine uNK cells, CD49b is also expressed by a small fraction of other cell types.¹³ To our knowledge, there is not an ideal marker for murine uNK cells. A cell purification strategy using DBA-lectin and CD122 marker may be more specific for mouse uNK cell purification.²² However, microbead-conjugated antibodies for DBA-lectin or CD122 are not commercially available. At present, microbead-conjugated anti-CD49b is used in MACS to purify uNK cells.²⁶ In future studies, it

would be helpful to exclude T cells and other cells by negative selection during uNK cell purification using microbead-conjugated CD3 and other antibodies specific for non-uNK cells that also express CD49b. Because B cells are virtually absent from the pregnant uterus, the possibility of B-cell contamination is small.²⁷ A recent report suggested that CD122 is a good marker for uNK cells.²² To define the percentage of CD122⁺ cells in the CD49b⁺ population, we performed two-color flow cytometry using MACS-purified CD49b⁺ cells stained with FITC-conjugated anti-CD122 and PE-conjugated anti-CD49b antibodies, which showed that most CD49b⁺ cells were also positive for CD122 in CBA/J×DBA/2J and CBA/J×BALB/c mice.

Flow cytometry is quantitative by nature, allowing thousands of cells to be counted and objectively analyzed within minutes. Using two-color flow cytometry with cells stained by FITC-conjugated anti-stathmin-1 and PE-conjugated anti-CD49b, we confirmed that there are double-positive cells that express stathmin-1 and CD49b and determined the constitutional ratio of these cells. Stathmin-1 expression in CD49b⁺ cells was also confirmed using MACS-purified CD49b⁺ cells and proteomic assays in the present study (Figures 2 and 3).

In summary, decreased stathmin-1 expression in a murine abortion-prone model was confirmed using flow cytometry, 2-DE, MS, and Western blot analysis compared with normal fertile controls. In multivision IHC, colocalization of stathmin-1 and DBA-lectin was confirmed in both matings, but the frequency of stathmin-1⁺DBA-lectin⁺ cells was significantly lower in abortion-prone matings. Inhibition of stathmin-1 significantly boosted embryo resorption rates in mouse models. In patients who experience unexplained spontaneous abortion, the frequency of stathmin-1⁺CD56⁺ cells was also significantly lower than in normal pregnancy. These results suggest that adequate stathmin-1 expression in uNK cells may be critical to pregnancy success. In contrast, insufficient stathmin-1 expression may be correlated with pregnancy failure.

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