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Insufficient Peroxiredoxin-2 Expression in Uterine NK Cells Obtained From a Murine Model of Abortion

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ABSTRACT

The CBA/J × DBA/2 mouse mating combination is prone to spontaneous embryo loss, in contrast to the MHC-identical CBA/J × BALB/c mating combination, which yields successful pregnancies. The underlying mechanisms for these observations are unclear. In this study, multi-vision immunohistochemical staining (IHC), flow cytometry and Western blot analysis were used to detect peroxiredoxin-2 (PRX-2) expression in the uterine natural killer (uNK) cells from CBA/J × DBA/2 and CBA/J × BALB/c mice. In IHC analysis, co-localization of PRX-2 and lectin from Dolichos biflorus agglutinin (DBA-lectin) was confirmed and the frequency of PRX-2$^+$DBA-lectin$^+$ cells was significantly lower in CBA/J × DBA/2 than CBA/J × BALB/c. In flow cytometry and Western blotting, PRX-2 was found expressed at a significantly lower level in CBA/J × DBA/2 mice. PRX-2 inhibition with a neutralizing antibody significantly decreased PRX-2 expression, increased the cytotoxicity of uNK cells, and increased the percentage of embryo loss in CBA/J × DBA/2J mice. Our data suggest that PRX-2 may be involved in the modulation of maternal–fetal tolerance and that insufficient expression of this protein may correlate with increased embryo loss in CBA/J × DBA/2J mice. J. Cell. Biochem. 112: 773–781, 2011.

KEY WORDS: ABORTION; ANIMAL MODEL; IMMUNE MODULATION; PREGNANCY; PROTEOMICS

CBA/J × DBA/2J (female CBA/J impregnated by male DBA/2J) crossed mice are commonly used as a mouse model of recurrent spontaneous abortion (RSA). In this model, a large number of healthy embryos that do not possess chromosome abnormalities are rejected and resorbed. The natural resorption rate of embryos from CBA/J × DBA/2J mice has been reported to be approximately 20–40%. This is significantly higher than that observed in the normal, fertile CBA/J × BALB/c crossed model, which is approximately 4–5%, despite both models' having an H-2^k × H-2^d MHC genotype [Clark et al., 1980; Lin et al., 2004].

Abbreviations used: AP-1, activator protein-1; CBA/J × BALB/c, female CBA/J mouse impregnated by male BALB/c; CBA/J × DBA/2J, female CBA/J mouse impregnated by male DBA/2J; DAPI, 4′,6-diamidino-2-phenylindole; DBA-lectin, lectin from Dolichos biflorus agglutinin; IHC, immunohistochemistry; MACS, magnetic affinity cell sorting; MFI, mean fluorescence intensity; MLAp, mesometrial lymphoid aggregate of pregnancy; NKEFB, NK cell-enhancing factor B; NOD, non-obese diabetic; PBS, phosphate-buffered solution; PE, phycoerythrin; PRX-2, peroxiredoxin-2; RSA, recurrent spontaneous abortion; SCID, severe combined immunodeficiency; TNF-α, tumor necrosis factor-α; uNK cell, uterine natural killer cell.

Guangjie Yin and Cui Li contributed equally to this study.

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Uterine natural killer (uNK) cells rapidly expand during endometrial decidualization and account for 70% of leukocytes in early gestational uteri of humans and rodents [Koopman et al., 2003; Trowsdale and Betz, 2006]. These cells have important functions for ensuring a successful pregnancy by having a critical role in embryo implantation and the maintenance of decidual tissue that supports placental and fetal development. A hypothesis postulates that uNK cells express molecules that are not shared by circulating NK cells or other leukocytes and therefore having a unique role that is distinct from normal circulating NK cells [Bizinotto et al., 2008]. However, it is also found that uNK cells and circulating NK cells share CD49b expression and it is also possible that a loss of uNK function may be compensated by infiltrating circulating NK cells [Lin et al., 2009b].

Peroxiredoxin-2 (PRX-2) is also known as NK cell-enhancing factor B (NKEFB) or thioredoxin peroxidase-1 [Zhang et al., 1997; Shau et al., 1998]. A recent study found that PRX-2 is expressed at a significantly lower level in non-obese diabetic (NOD) mice. These mice have a deficiency in NK cell function compared to wild-type mice and the lower level of PRX-2 expression is thought to be associated with increased embryo loss in pregnant NOD mice [Li et al., 2009]. In addition, NK cell abnormality has been observed in CBA/J × DBA/2J mice [Clark et al., 1998]. However, it is unclear whether PRX-2 is expressed in uNK cells derived from CBA/J × DBA/2J mice or if PRX-2 has a mechanistic role in allogeneic pregnancy tolerance.

In this study, the distribution pattern of PRX-2$^+$ cell subset within uNK cells from pregnant female mice was detected by multi-vision immunohistochemistry (IHC), and uNK cells were purified from both CBA/J × DBA/2J and CBA/J × BALB/c allogeneic pregnant models. The percentage of PRX-2$^+$ cells in the uNK cell population was detected by flow cytometry and the expression level of the protein in uNK cell lysates was detected by Western blot analysis. A possible role of PRX-2 in allogeneic pregnancy tolerance is discussed below.

MATERIALS AND METHODS

PREGNANT MODELS OF CBA/J × DBA/2J AND CBA/J × BALB/C CROSSED MICE

Female CBA/J mice, male DBA/2J, and BALB/c mice (8–12 weeks old) were purchased from the Model Animal Center of Nanjing University (Nanjing, China), and housed under specific pathogen-free conditions. All animal procedures followed the national animal care guidelines and all associated data were approved for publication by the University's Institutional Review Board. Abortion (resorption) sites were identified by their small size, accompanied by a necrotic, hemorrhagic appearance relative to normal embryos and placentas. The percentage of resorptions was calculated as the ratio of resorption sites and total implantation sites (resorption plus normal implantation sites), as described previously [Zenclussen et al., 2006; Lin et al., 2009b].

MULTI-VISION IHC ANALYSIS OF PLACENTAL TISSUE

Placentas together with adjacent decidual tissues were collected on embryonic day E10.5 from CBA/J × DBA/2J and CBA/J × BALB/c mice and used to measure the distribution and abundance of the protein PRX-2 by multi-vision IHC procedure [Lin et al., 2005b; Zhang et al., 2009]. Paraffin blocks were cut into 4-μm-thick sections. The sections were then deparaffinized in xylene and rehydrated in graded alcohol concentrations. Goat serum was added onto all the sections for 30 min to minimize the possibility of primary antibody uptake artifacts associated with the preparation of tissue samples. Non-specific binding was further blocked by preincubation with blocking solution for 5 min followed by incubation 1 h at 4°C with rabbit anti-mouse PRX-2 (1:200 dilution) (Cell Signaling Technology, Inc., Beverly, MA). Meanwhile, FITC-conjugated lectin from Dolichos biflorus (DBA-lectin) was added onto the section (1:200 dilution) (Sigma-Aldrich, St. Louis, MO) in the dark [Zhang et al., 2009]. The sections were then washed three times with phosphate-buffered solution (PBS) for 5 min each and then incubated with phycoerythrin (PE)-conjugated anti-rabbit IgG (1:200 dilution) (Alpha Diagnostic International, San Antonio, TX) for 30 min at room temperature in the dark. Then 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, San Diego, CA) was used to stain nuclei for 10 min in the dark. Negative controls were established using rabbit Ig of the isotype identical to the rabbit anti-mouse primary antibody in place of the specific primary antibody (Cell Signaling Technology, Inc.).

PURIFICATION OF UTERINE NK CELLS

Cell purification was performed by magnetic affinity cell sorting (MACS). In brief, hysterolaparotomy was performed on embryonic day E12.5 to collect embryo-depleted placentas. The pooled placentas, together with the decidua basalis, were carefully cut into small pieces, collected in 0.9% NaCl solution, and subsequently filtered through a 50 μm pore size nylon mesh to obtain a single cell suspension. Mononuclear cells were obtained by centrifugation of the single cell suspension using a Ficoll-Hypaque density column. Any red blood cells that contaminated the single-cell suspension were eliminated by incubation with red blood cell lysis buffer (eBioscience, San Diego, CA) two times at 37°C. Subsequently, NK cells were isolated using magnetic bead-conjugated anti-mouse CD49b monoclonal antibody and CD49b$^+$ cells were purified using Mini MACS columns (Miltenyi Biotec., Auburn, CA) [Arase et al., 2001; Lin et al., 2008, 2009b]. The purity of the MACS-purified NK cells routinely exceeded 95% as determined by flow cytometry [Lin et al., 2008, 2009b].

FLOW CYTOMETRY

Uterine NK cells were collected on E8.5 and E12.5, purified by MACS, and stained with PE-conjugated anti-mouse CD49b (BioLegend, San Diego, CA) at a concentration of 10^6 cells/1 μg antibody, washed once with PBS, resuspended and incubated in permeabilization buffer (eBioscience) at a concentration of 10^6 cells/ml buffer for 10 min at 4°C, and then incubated with rabbit anti-PRX-2 (Abcam, Cambridge, UK) antibody at a concentration of 10^6 cells/1.5 μg antibody, followed by FITC-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) (10^6 cells/1 μg antibody) [Lin et al., 2009b]. The percentage of PRX-2$^+$ cells within the CD49b$^+$ NK cell population and mean fluorescence intensity (MFI) of PRX-2$^+$CD49b$^+$ cells were measured with a FACS Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ) [Lin et al., 2009b].

Isotype control antibodies (BioLegend) were used to exclude false-positive cells [Lin et al., 2006]. All experiments were independently performed four times [Li et al., 2006; Shen et al., 2007].

PREPARATION OF UTERINE NK CELL LYSATES

Uterine NK cells were suspended in a modified RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM ethylendiaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.66 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, 1 mM NaF) and sonicated three times in 5 s bursts. The cell lysates were centrifuged at 14,000*g* for 15 min at 4°C. The supernatants were collected and their protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA) [DiGiovanni et al., 2000].

WESTERN BLOT ANALYSIS

Aliquots of tissues were homogenized to powder using liquid nitrogen and dissolved in lysis buffer (150 mM NaCl, 50 mM Tris–Cl, pH 8.0, 0.1% nonidet 40 (NP-40), 1 mM PMSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin), vortexed, and incubated at room temperature for 2 h. The mixture was then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was used as the total protein solution. The concentration of the lysate was assayed with the Bradford assay (Bio-Rad Laboratories). Western blotting analysis was performed as previously described [Li et al., 2009]. Briefly, 100 μg of total protein was separated on a 12% SDS–PAGE gel prior to transfer onto a nitrocellulose membrane. After blocking with 5% milk in TBS/0.2% Tween 20 (TBS-T) for 1 h at room temperature, the membrane was incubated with rabbit anti-mouse PRX-2 antibody (1: 100 dilution) (Cell Signaling Technology, Inc.) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:10,000 dilution, Amersham Biosciences) for 1 h at room temperature. Detection of NADPH was used as loading control. The reactions were visualized using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences). Signals on the blots were visualized by autography.

EFFECT OF PRX-2 BLOCKING

PRX-2 inhibition was performed in both CBA/J × DBA/2J and CBA/J × BALB/c mice by intraperitoneal (i.p.) injection of anti-PRX-2 antibody (Cell Signaling Technology, Inc.) at embryonic days E4.5, E5.5, and E6.5, at a dosage of 40, 80, and 160 ng (0.2, 0.4, and 0.8 μg/ml in 0.2 ml of PBS) once a day, respectively. 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 at day E12.5 using the method described above.

STANDARD ^{51}CR RELEASE ASSAY

Under sterile conditions, uterine CD49b$^+$ cells were harvested on embryonic day E12.5 and purified by MACS from both CBA/J × DBA/2J and CBA/J × BALB/c mice pretreated with anti-PRX-2 on E4.5, E5.5, and E6.5, at a dosage of 80 ng (0.4 μg/ml in 0.2 ml of PBS) or equal volume of isotype control antibody once a day, and used as NK cell effectors. The cells were titrated twofold on 96-well plates and ^{51}Cr-labeled (Perkin-Elmer, Boston, MA) YAC-1 cells (5×10^3 cells per well; from American Type Culture Collection, Rockville, MD) were added and used as target cells. Each assay was performed in triplicate. After 4 h of incubation, the percentage of specific lysis was measured by a γ counter and calculated by the following formula: percentage specific lysis = (experimental c.p.m. − spontaneous release c.p.m.)/(total c.p.m. − spontaneous release c.p.m.) × 100. Total count per minute (c.p.m.) was determined from the wells receiving ^{51}Cr-labeled YAC-1 target cells and 2.5% Triton X-100 (Sangon, Shanghai, China) [Lin et al., 2005a, 2009a]. The PRX-2 protein level upon anti-PRX-2 pretreatment or isotype control antibody was determined by Western blot analysis, as described above.

STATISTICAL ANALYSIS

Flow cytometry data were analyzed using Quad statistics and the cell percentages derived from flow cytometric analysis were further compared using independent sample *t*-test among groups [Lin et al., 2006]. The mean density of positive cells in IHC and that of PRX-2 protein level in Western blot analysis were analyzed using Image-Pro Plus 6.0 software package and compared using independent sample *t*-test among groups. Results were expressed as means ± standard deviation. The resorption rate of embryos and NK cell cytotoxicity was compared using χ^2 test [Li et al., 2006; Shen et al., 2007]. Experiments were repeated independently as indicated in figure legends.

RESULTS

PERCENTAGE OF EMBRYO LOSS

The percentage of resorbed embryos at embryonic day E12.5 was 24.6% ($n = 12$; 35 of 142) in CBA/J × DBA/2J crossed mice and 3.2% ($n = 16$; 6 of 187) in CBA/J × BALB/c mice. The resorption rate was significantly higher in CBA/J × DBA/2J crossed mice ($P < 0.01$) and supports the hypothesis that these mice are prone to spontaneous embryo loss.

MULTI-VISION IHC PATTERN OF PRX-2 EXPRESSION

Multi-vision IHC analysis showed that the cells positive for DBA-lectin were scattered throughout tissues near the interface where the placenta is attached to the uterus. Intensive staining was detected in the decidua basalis and mesometrial lymphoid aggregate of pregnancy (MLAp) in both CBA/J × DBA/2J and CBA/J × BALB/c mice, in agree with the distribution pattern of uNK cells (Fig. 1A,B). The DBA-lectin$^+$ cells were dominant compared with PRX-2$^+$ cells in both matings. Among the cells positive for DBA lectin, a fraction was also positive for PRX-2 (Fig. 1, C4, D4, E4, F4, C8, D8, E8, F8). These double positive cells were yellow colored in the merged images (C4, D4, E4, F4, C8, D8, E8, F8). The frequency of PRX-2$^+$ cells within DBA-lectin$^+$ population was significantly lower in CBA/J × DBA/2J mice than CBA/J × BALB/c mice (C4, D4, E4, F4, C8, D8, E8, F8). The overall density of PRX-2$^+$ cells in the sections from CBA/J × DBA/2J mice was significantly lower than that in CBA/J × BALB/c mice (1.0 ± 0.7 vs. 6.9 ± 4.2, $P < 0.01$) (Fig. 1G).

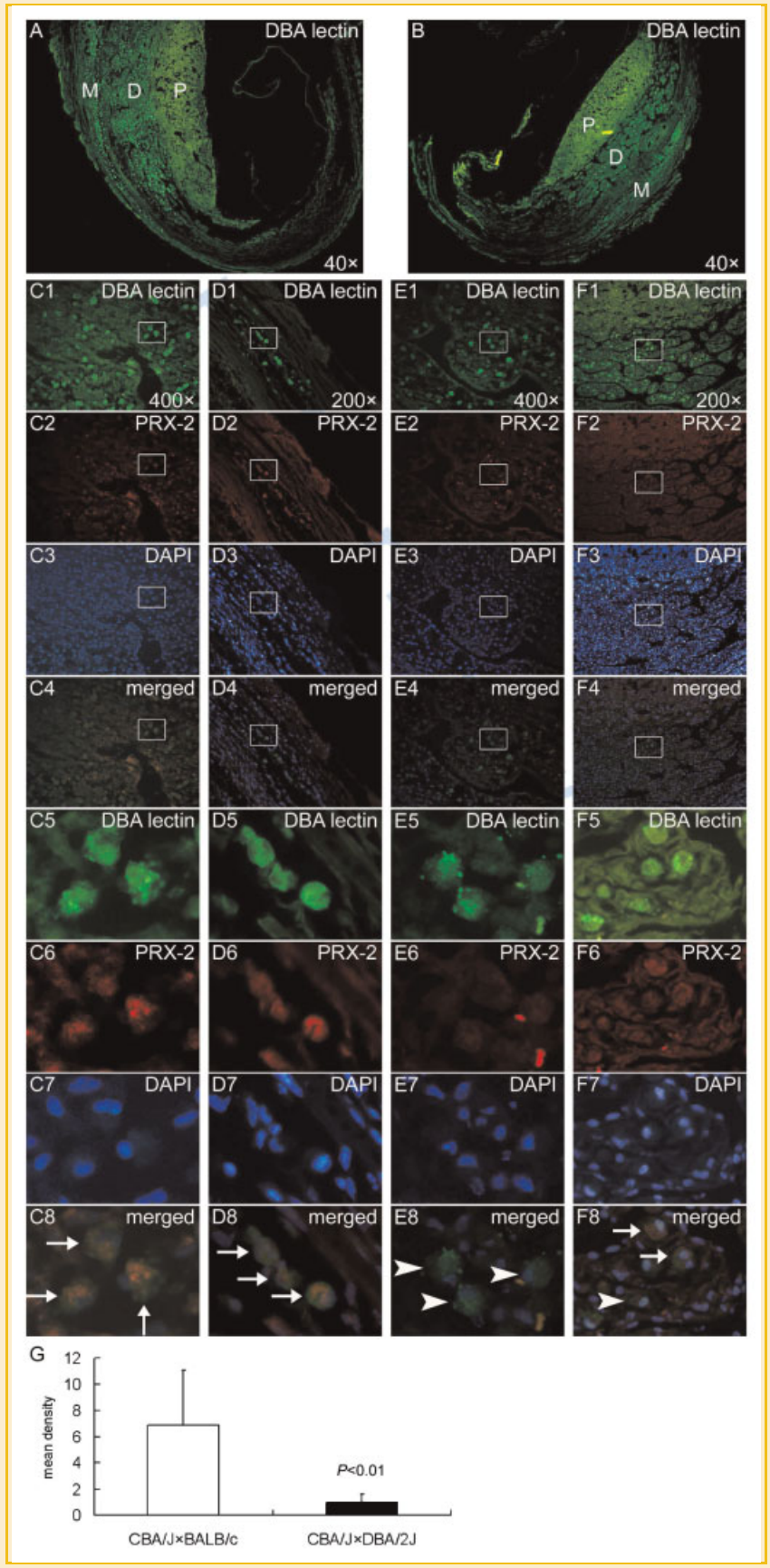
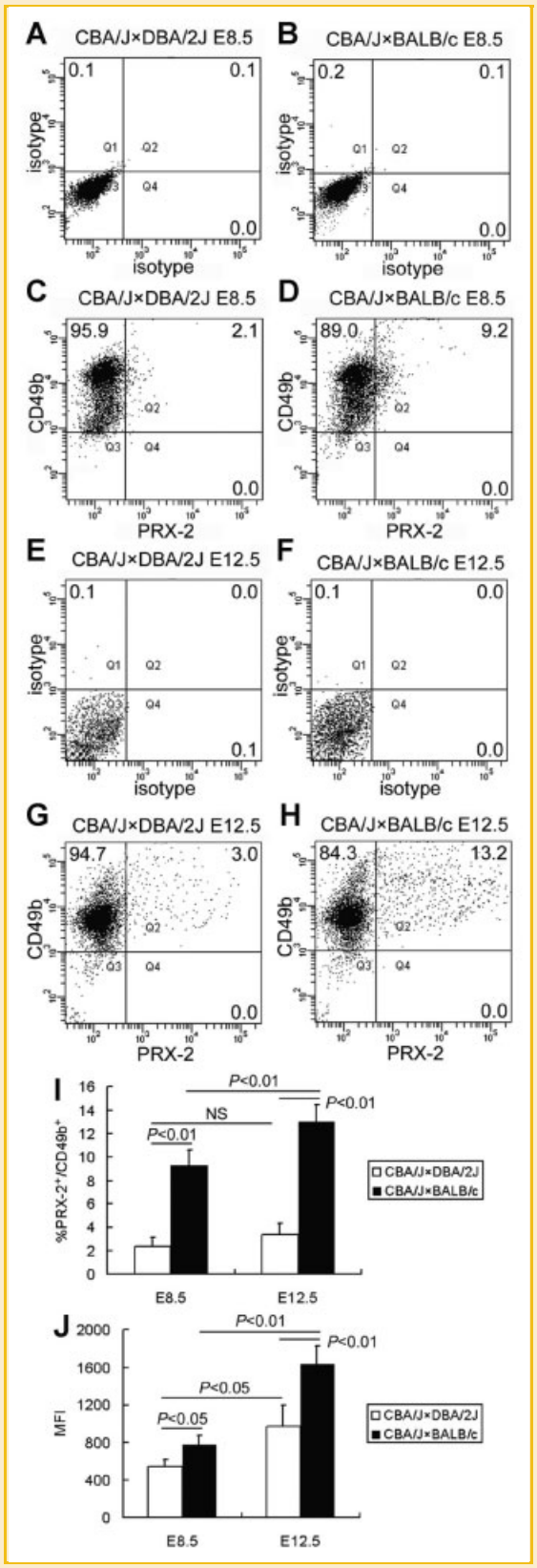


Fig. 1. Multi-vision immunohistochemical analysis of PRX-2 expression in placenta and decidua. A, C1–C8 and D1–D8: From CBA/J × BALB/c mice. B, E1–E8 and F1–F8: From CBA/J × DBA/2J mice. G: Mean density of PRX-2$^+$DBA-lectin$^+$ cells as determined by Image-Pro Plus 6.0. Mouse uterine NK cells were stained for DBA lectin (green) and PRX-2 (red) co-localization. Nuclei were stained using DAPI (blue). Original magnification was indicated. A,B: M, MLAp; D, decidual basalis; P, placenta. In both mating combinations, DBA-lectin$^+$ cells intensively scattered in the regions M and D, whereas no DBA-lectin$^+$ cells were found in region P. C5–C8, D5–D8, E5–E8, F5–F8: Local magnification of the region indicated by the box on C1–C4, D1–D4, E1–E4, F1–F4, respectively. In the merged images, PRX-2$^+$DBA-lectin$^+$ NK cells (yellow) were indicated with arrows, and PRX-2$^-$ NK cells (green) were indicated with arrowheads (C8, D8, E8, F8). The frequency of PRX-2$^+$DBA-lectin$^+$ cells (C4, D4, E4, F4, C8, D8, E8, F8) and the mean density of these cells (G) was significantly lower in CBA/J × DBA/2J mice than CBA/J × BALB/c mice (1.0 ± 0.7 vs. 6.9 ± 4.2; $P < 0.01$).



FLOW CYTOMETRY

The existence of PRX-2 and CD49b double-positive (PRX-2^+CD49b^+) cells was confirmed by two-color flow cytometry and the percentage of PRX-2^+CD49b^+ cells within CD49b^+ NK cell population was significantly lower in CBA/J × DBA/2J mice than CBA/J × BALB/c mice (on E8.5, 2.4 ± 0.7% vs. 9.3 ± 0.8%; $P < 0.01$; on E12.5, 3.4 ± 1.0% vs. 13.0 ± 1.5%; $P < 0.01$) (Fig. 2). This was consistent with the results of IHC staining. In CBA/J × DBA/2J mice, the percentage of PRX-2^+CD49b^+ cells within CD49b^+ population on E8.5 was not significantly different from that detected on E12.5 (2.4 ± 0.7% vs. 3.4 ± 1.0%; $P > 0.05$). In contrast, the same percentage in CBA/J × BALB/c mice detected on E8.5 was significantly lower than that on E12.5 (9.3 ± 0.8% vs. 13.0 ± 1.5%; $P < 0.01$).

In CBA/J × DBA/2J mice, the MFI of PRX-2^+CD49b^+ cells on E8.5 and E12.5 was 546 ± 90 and 772 ± 128, both significantly lower than the corresponding MFI in CBA/J × BALB/c mice (974 ± 225 on E8.5, $P < 0.05$; 1,633 ± 200 on E12.5, $P < 0.01$), respectively. In addition, the MFI on E8.5 was significantly lower than on E12.5 in both mating combinations: 546 ± 90 versus 772 ± 128 in CBA/J × DBA/2J mice ($P < 0.05$) and 974 ± 225 versus 1,633 ± 200 in CBA/J × BALB/c mice ($P < 0.01$), respectively.

VERIFICATION OF PRX-2 EXPRESSION BY WESTERN BLOT ANALYSIS

Western blot analysis was performed on uNK cell lysates isolated from CBA/J × DBA/2J mice and age-matched CBA/J × BALB/c mice at embryonic day E12.5 (Fig. 3). The level of PRX-2 protein expression was significantly higher in CBA/J × BALB/c mice than CBA/J × DBA/2J mice.

EFFECT OF PRX-2 INHIBITION ON EMBRYO LOSS

As shown in Figure 4, the percentage of embryo resorption was significantly higher in CBA/J × DBA/2J mice (52.3 ± 25.7% (23/44) in the blocked group vs. 26.3 ± 17.2% (15/57) in the isotype control antibody group; $P < 0.01$) when PRX-2 protein was inhibited by a neutralizing antibody at a dosage of 40 ng, daily for 3 days. The change in the percentage of embryos lost in CBA/J × BALB/c mice treated with a neutralizing antibody at the same dosage did not reach statistical significance (17.3 ± 14.7% (9/52) in the blocked group vs. 10.3 ± 6.8% (6/58) in the isotype control antibody group; not significant). When the dosage of neutralizing antibody was increased to 80 ng, the resorption rate was further increased in both mating combinations and significantly higher than the corresponding isotype control antibody groups and the 40-ng dosage groups (58.2 ± 12.5% (32/55) for CBA/J × DBA/2J and 26.3 ± 8.6% (15/57) for CBA/J × BALB/c; $P < 0.01$ for all). However, the percentage of

Fig. 2. Flow cytometric analysis of PRX-2^+ cells in uterine CD49b^+ cell population. A–H: Representative results. A,B,E,F: Isotype controls. E8.5, embryonic day 8.5. E12.5, embryonic day 12.5. I: Data summary of the PRX-2^+ cell percentage in CD49b^+ population. J: Mean fluorescence intensity (MFI) of PRX-2^+CD49b^+ cells. Experiments were repeated four times for each group. NS, not significant.

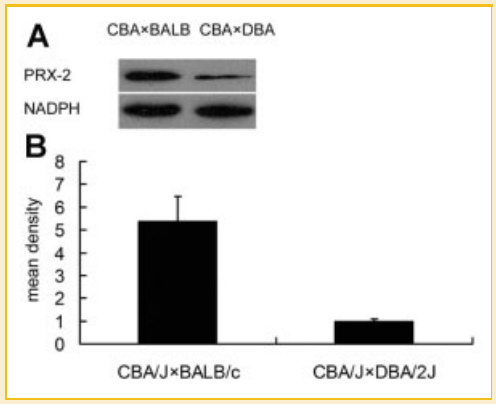


Fig. 3. Western blot analysis of PRX-2 expression. CBA × BALB, CBA/J × BALB/c. CBA × DBA, CBA/J × DBA/2J. A: The expression of PRX-2 was significantly lower in the CBA/J × DBA/2J mice than the CBA/J × BALB/c mice. NADPH was used as an internal loading control. B: Histogram shows the relative expression level of PRX-2 protein in CBA/J × BALB/c and CBA/J × DBA/2J mice. Identical experiments were repeated four times for each group.

embryo lost kept unchanged when the dosage was increased to 160 ng (58.5 ± 12.1% (31/53) for CBA/J × DBA/2J and 26.7 ± 8.9% (16/60) for CBA/J × BALB/c; not significant when compared with the corresponding 80-ng dosage groups, and $P < 0.01$ for all when compared with the corresponding no-inhibition groups and 40-ng dosage groups).

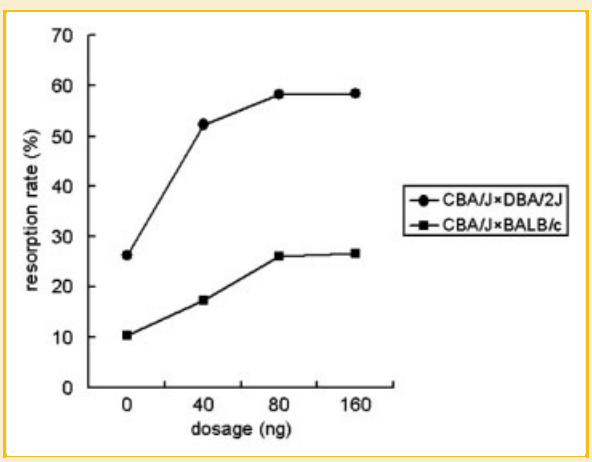


Fig. 4. Effect of PRX-2 inhibition on embryo resorption. CBA/J × DBA/2J and CBA/J × BALB/c mice injected with the same volume and frequency of isotype control antibody was defined as dosage "0." The percentage of embryo lost on embryonic day E12.5 was markedly increased in CBA/J × DBA/2J mice when PRX-2 neutralizing antibody was injected at a dosage of 40 ng daily for 3 days, and further increased if the antibody was injected at a dosage of 80 ng. However, the resorption rate was not further increased if the dosage increased to 160 ng. In CBA/J × BALB/c mice, the resorption rate was not significantly increased if PRX-2 neutralizing antibody was injected at a dosage of 40 ng, but marked increased at a dosage of 80 ng and kept unchanged at the higher level if the antibody was injected at a dosage of 160 ng. Six mice were examined at each dosage in each mating combination.

EFFECT OF PRX-2 BLOCKADE ON NK CELL CYTOTOXICITY AND PRX-2 PROTEIN EXPRESSION

A significantly higher cytolytic activity could be observed in uterine $CD49b^+$ cells purified by MACS from isotype control antibody-treated CBA/J × DBA/2J mice, as compared with that from isotype control antibody-treated CBA/J × BALB/c mice. All the mean value of specific lysis percentage at each effector/target ratio were significantly higher in CBA/J × DBA/2J mice than the corresponding value detected in CBA/J × BALB/c mice ($P < 0.01$ in χ^2 test) (Fig. 5A). The cytotoxicity was significantly increased upon anti-PRX-2 treatment in both mating combinations ($P < 0.01$ for both) (Fig. 5A). Decreased PRX-2 protein expression was confirmed by Western blot analysis in both mating combinations pretreated with anti-PRX-2 antibody. The mean density was 4.9 ± 1.8 in CBA/J × BALB/c matings pretreated with isotype control antibody, significantly higher than the same matings pretreated with anti-PRX-2 (0.7 ± 0.3; $P < 0.01$). The mean density was 1.0 ± 0.4 in CBA/J × DBA/2 matings pretreated with isotype antibody, significantly higher than the same matings pretreated with anti-PRX-2 (0.2 ± 0.1; $P < 0.01$). The mean density in CBA/J × BALB/c matings pretreated with isotype antibody was also significantly higher than CBA/J × DBA/2 matings pretreated with isotype antibody ($P < 0.01$) (Fig. 5B,C).

DISCUSSION

PRX-2, originally cloned as NKEFB, belongs to a highly conserved antioxidant family and is involved in redox regulation of cells [Lim et al., 1998; Carninci et al., 2005]. Tumor necrosis factor-α (TNF-α) activates the expression of activator protein-1 (AP-1) responsive genes through the induction of reactive oxygen species (ROS). PRX-2 may protect endothelial cells from being damaged through the inhibition of TNF-α-induced ROS [Shau et al., 1998]. In addition, TNF-α-mediated induction of ROS can result in abortion, and therefore inhibition of TNF-α by PRX-2 may be extremely important for the maintenance of proper blood supply exchange between the uterus and early-stage embryo.

It is widely believed that some NK cell subsets play critical roles in the establishment and maintenance of pregnancy [Croy et al., 2002; Lin et al., 2006]. Based on NK cell deficiency models, previous studies have found that an absence of NK cells during pregnancy is associated with reproductive deficits [Guimond et al., 1997, 1998; Lin et al., 2006]. Additionally, depletion of residual NK cells in NOD/severe combined immunodeficiency (SCID) mice enhances the percentage of embryos lost [Shultz et al., 2003; Lin et al., 2005a]. Our recent study found that the level of PRX-2 expression was significantly lower in NK cell-deficient NOD mice compared to wild-type controls. The level of PRX-2 may therefore correlate with the impaired fertility in NOD mice [Li et al., 2009].

In the present study, microbead-conjugated anti-CD49b and a MiniMACS system is used to isolate murine uNK cells from CBA/J × DBA/2J and CBA/J × BALB/c matings [Arase et al., 2001]. Since CD49b is also expressed by a small fraction of other cells, such as B cells, there is a chance that our preparation of NK cells is contaminated with minor population of other cell types [Arase et al.,

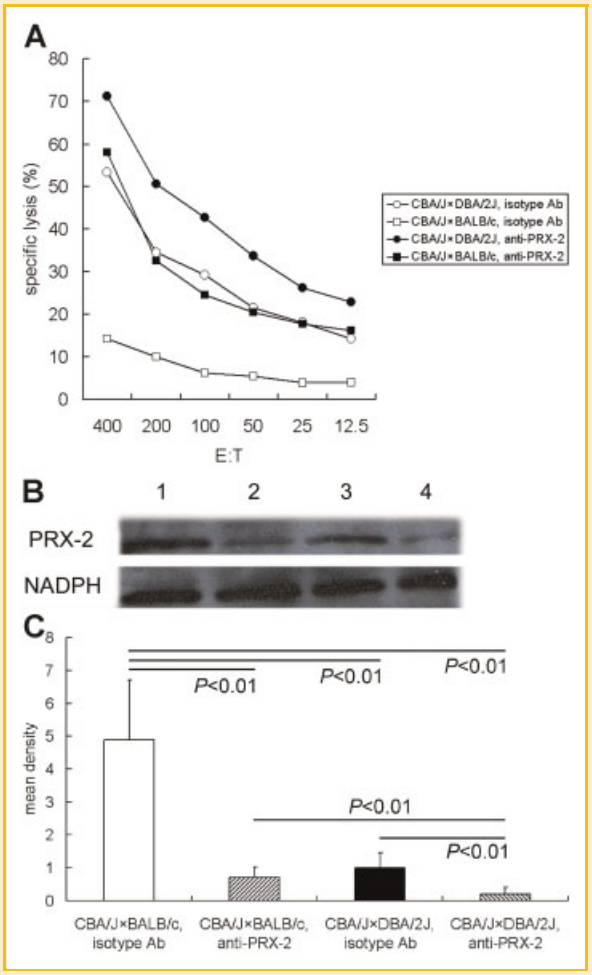


Fig. 5. Effect of PRX-2 blockade on uterine NK cell cytotoxicity and PRX-2 expression. A: PRX-2 was inhibited with multiple injection of anti-PRX-2 or not inhibited with multiple injection of isotype antibody (Ab). NK cell cytotoxicity was detected by ^{51}Cr release assay. E:T, effector/target ratio. Uterine CD49b$^+$ cells purified by MACS on embryonic day E12.5 were used as effector cells. ^{51}Cr-labeled YAC-1 cells were used as target cells. The cytotoxicity was significantly higher in uterine CD49b$^+$ cells from CBA/J × DBA/2J mice pretreated with isotype Ab than that from CBA/J × BALB/c mice pretreated with isotype Ab ($P < 0.01$ for all the specific lysis percentages at each E:T ratio). The cytotoxicity was increased in both mating combinations after pretreated with anti-PRX-2 Ab ($P < 0.01$ for both). B,C: Confirmation of decreased PRX-2 expression after pretreated with anti-PRX-2 Ab in uNK cells from both mating combinations in Western blot analysis. B: Representative Western blot analysis. Lane 1: CBA/J × BALB/c, pretreated with isotype Ab. Lane 2: CBA/J × BALB/c, pretreated with anti-PRX-2. Lane 3: CBA/J × DBA/2J, pretreated with isotype Ab. Lane 4: CBA/J × DBA/2J, pretreated with anti-PRX-2. C: Histogram shows the relative expression level of PRX-2 protein in CBA/J × BALB/c and CBA/J × DBA/2J mice pretreated with anti-PRX-2 or isotype Ab. Identical experiments were repeated four times for each group. PRX-2 production was significantly decreased in both mating combinations after PRX-2 blockade ($P < 0.01$ for both). In addition, PRX-2 level was significantly lower in both CBA/J × DBA/2J groups pretreated with isotype Ab and anti-PRX-2 Ab than the corresponding CBA/J × BALB/c groups ($P < 0.01$ for both).

2001]. However, as an ideal marker for NK cells does not exist at present, CD49b is commonly used to isolate uNK cells in murine models [Arase et al., 2001; Wang et al., 2009]. Because NK cells are the most abundant type of leukocytes in the pregnant uterus till mid-gestation, while B cells are virtually absent, the possibility of B cell contamination is very small [von Rango et al., 2001]. Dolichus biflores agglutinin (DBA) lectin is possibly more specific for murine uNK cell sorting [Yadi et al., 2008]. However, there is not commercially available microbead-conjugated anti-DBA antibody. In future studies, it will surely be helpful to exclude other cells by negative selection during uNK cell purification using microbead-conjugated antibodies.

In the present study, FITC-conjugated DBA lectin was used to confirm PRX-2 localization within uNK cells in multi-vision IHC analysis. DBA-lectin$^+$ cells were dominant in MLAp and decidual basalis in IHC, suggesting that DBA lectin is a relatively ideal pan-marker for uNK cells. In the merged images, the presence of PRX-2$^+$stathmin-1$^+$ cells was confirmed and these double positive cells were shown as yellow-colored cells. In addition, the frequency of PRX-2$^+$stathmin-1$^+$ cells was significantly lower in CBA/J × DBA/2J matings than CBA/J × BALB/c matings. This support the hypothesis that PRX-2 molecule is not sufficiently expressed in abortion-prone CBA/J × DBA/2J matings.

In our current study, the CBA/J × DBA/2J mating combination was confirmed to be prone to spontaneous embryo loss. The natural resorption rate of this model was significantly higher than the CBA/J × BALB/c controls and consistent with previous studies [Clark et al., 1998; Lin et al., 2004]. Furthermore, our current study showed that PRX-2 expression level was significantly lower in the CBA/J × DBA/2J mating combination. This observation was supported by IHC, flow cytometry, and Western blot analysis: the mean density of PRX-2$^+$ NK cells in IHC was significantly higher in CBA/J × BALB/c mice than in CBA/J × DBA/2J mice. The existence of PRX-2$^+$ NK cells was further confirmed by two-color flow cytometry. Consistent with the IHC results in our present study, the percentage of PRX-2$^+$ cells in the uNK cell population determined by flow cytometry was significantly lower in CBA/J × DBA/2J mice than the control CBA/J × BALB/c mice on both E8.5 and E12.5. This suggests that PRX-2 was insufficiently expressed in CBA/J × DBA/2J mice at both an earlier stage and mid-stage of gestation. Similar results were obtained in MFI analysis: The MFI of PRX-2$^+$CD49b$^+$ cells in CBA/J × DBA/2J mice was significantly lower than CBA/J × BALB/c mice on both E8.5 and E12.5.

In addition, the percentage of PRX-2$^+$CD49b$^+$ cells in the uNK cell population was significantly higher on E12.5 than on E8.5 in both mating combinations. The same trend was also observed in MFI analysis. These results suggest that PRX-2 expressed at a higher level at mid-stage than at early stage of gestation. This is concomitant with the higher frequency of uNK cells on E12.5 than on an earlier timepoint of gestation. In future, it is considerable to make clear whether the PRX-2 level on a later timepoint of gestation was lower than on E12.5. Notably, Western blot analysis confirmed the results in double-vision IHC and flow cytometry: the mean density of PRX-2 bands from uNK cell lysates of CBA/J × BALB/c mice was significantly higher than CBA/J × DBA/2J mice. These results further support the hypothesis that PRX-2 may be insufficiently expressed in abortion-prone matings.

When PRX-2 neutralizing antibody was injected at a dosage of 40 ng at embryonic days E4.5, E5.5, and E6.5, the resorption rate significantly increased only in CBA/J × DBA/2J mice, but

not in CBA/J × BALB/c mice. This result suggests that CBA/J × DBA/2J mice seemed to be more sensitive to anti-PRX-2 treatment than CBA/J × BALB/c mice. Moreover, when the dosage of PRX-2 neutralizing antibody increased from 40 to 80 ng, the resorption rates increased markedly in both mating combinations. In contrast, the resorption rates did not increase further when the dosage increased from 80 to 160 ng, suggesting that a status of saturation has been reached at a dosage between 80 and 160 ng, and that the injected antibody almost fully neutralized the PRX-2 at a dosage of 80 ng [Lin et al., 2006]. These results suggest that the maternal immune system is much more sensitive to anti-PRX-2 antibody treatment in CBA/J × DBA/2J than CBA/J × BALB/c matings, and that insufficient PRX-2 expression is much likely to be a "cause," rather than a "secondary result" of embryo resorption.

The efficacy of neutralizing antibodies to block PRX-2 is likely to be greatly confounded by pharmacological variables including clearance of the antibody, tissue distribution, uptake and saturation. In our present study, PRX-2 blockade was confirmed by Western blot analysis: upon anti-PRX-2 treatment, the density of PRX-2 bands in Western blotting was significantly decreased in both mating combinations. In contrast, no such trends were observed in isotype control antibody treated matings. In future studies, PRX-2 knock-out or knock-in models will be more effective to clarify PRX-2 function in the uterus during pregnancy. Also, it is desirable to use PRX-2 knock-in model or performing PRX-2 restoration experiment and investigate subsequent changes of pregnancy outcomes in CBA/J × DBA/2J mice.

Uterine NK cells are heterogenous. It consists of NK1, NK2, NK3, and NKr1 subsets categorized by cytokine profile [Higuma-Myojo et al., 2005; Lin et al., 2009a]. NK1 subset mainly expresses IFN-γ and TNF-α, NK2 subset mainly expresses IL-4, NK3 subset mainly produces TGF-β, and NKr1 subset mainly produces IL-10 [Higuma-Myojo et al., 2005; Lin et al., 2009a]. In our present study, approximately 13% of the uterine $CD49b^+$ cells from CBA/J × BALB/c mice express PRX-2, while approximately 3% of the uterine $CD49b^+$ cells from CBA/J × DBA/2J mice express this molecule in flow cytometric analysis. It suggests that $PRX\text{-}2^+$ cells may be a specific subset of mouse uNK cells.

Physiologically, the cytotoxicity of uterine NK cells is significantly lower than that of circulating NK cells [Lin et al., 2009a]. In our present study, the cytotoxicity of MACS-purified $CD49b^+$ uNK cells from CBA/J × DBA/2J mice was significantly higher than that from CBA/J × BALB/c mice. Since a significantly higher percentage of $PRX\text{-}2^+$ fraction was detected within the $CD49b^+$ cells from CBA/J × BALB/c mice than that from CBA/J × DBA/2J mice, it seems that a higher PRX-2 level is correlated with a lower level of cytotoxicity. This was supported by the results that anti-PRX-2 treatment significantly increased the cytotoxicity of MACS-purified $CD49b^+$ cells in both mating combinations as compared with the mice pretreated with isotype control antibody. These results suggest that PRX-2 may play a critical role in the modulation of NK cell cytotoxicity. Increased spontaneous embryo-resorption rate in CBA/J × DBA/2J mice may be partially attributed to insufficient PRX-2 expression and increased cytotoxicity of uNK cells in these mice [Lin et al., 2005a, 2009a].

In future researches, it is desirable to perform buffer or saline solution perfusion through the vascular bed of animals before collecting and sampling the uterus-placenta tissue, aiming to avoid circulating blood cell contamination and assure all the experimental results are from uterus-placenta tissue. Notably, although some researchers suggest that uNK cells express molecules that are not shared by circulating NK cells or other leukocytes and therefore having a unique role that is distinct from normal circulating NK cells [Bizinotto et al., 2008], our recent study found that uNK cells and circulating NK cells share CD49b and other molecule expression and a partially loss of uNK function may be compensated by circulating NK cells: intravenous adoptive transfer of $ITGA2^+ISG20^+$ ($CD49b^+CD25^+$) cells significantly decreased the percentage of embryo lost in NK cell-deficient NOD mice, and CXCL12–CXCR4 interaction may participate in the migration of circulating NK cells into pregnant uterus [Lin et al., 2009b].

PRX-1 is the first described member of the peroxiredoxin family. Mice lacking PRX-1 are viable and fertile but have a shortened lifespan due to the development of severe hemolytic anemia and several malignant cancers at approximately 9 months of age [Neumann et al., 2003]. PRX-1-null mice have abnormalities in the number, phenotype, and function of NK cells [Neumann et al., 2003]. The function of PRX-2 is somewhat different from PRX-1. Previous studies suggest that PRX-2 enhance NK cell function and protect DNA and proteins from oxidative damage [Zhang et al., 1997; Shau et al., 1998]. The preferential distribution of $PRX\text{-}2^+$ NK cells in the decidua basalis and tissues adjacent to blood vessels implies that this protein may be involved in the establishment of maternal–fetal blood supply and the regulation of immune cell function. The mechanism in detail is to be explored in further researches.

In summary, the findings in our present study suggest that the expression pattern of PRX-2 in uNK cells may be associated with the functional status of these cells in CBA/J × DBA/2J mice and other murine models especially those suffer from NK cell deficiency or abnormality [Clark et al., 1998; Li et al., 2009; Lin et al., 2009b]. Also, insufficient PRX-2 expression by uNK cells may be associated with some cases of unexplained RSA and other kinds of pregnancy-related diseases in human. However, this need to be confirmed and further investigated in future comparative researches using the decidual samples from RSA patients and healthy elective pregnancy termination.

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