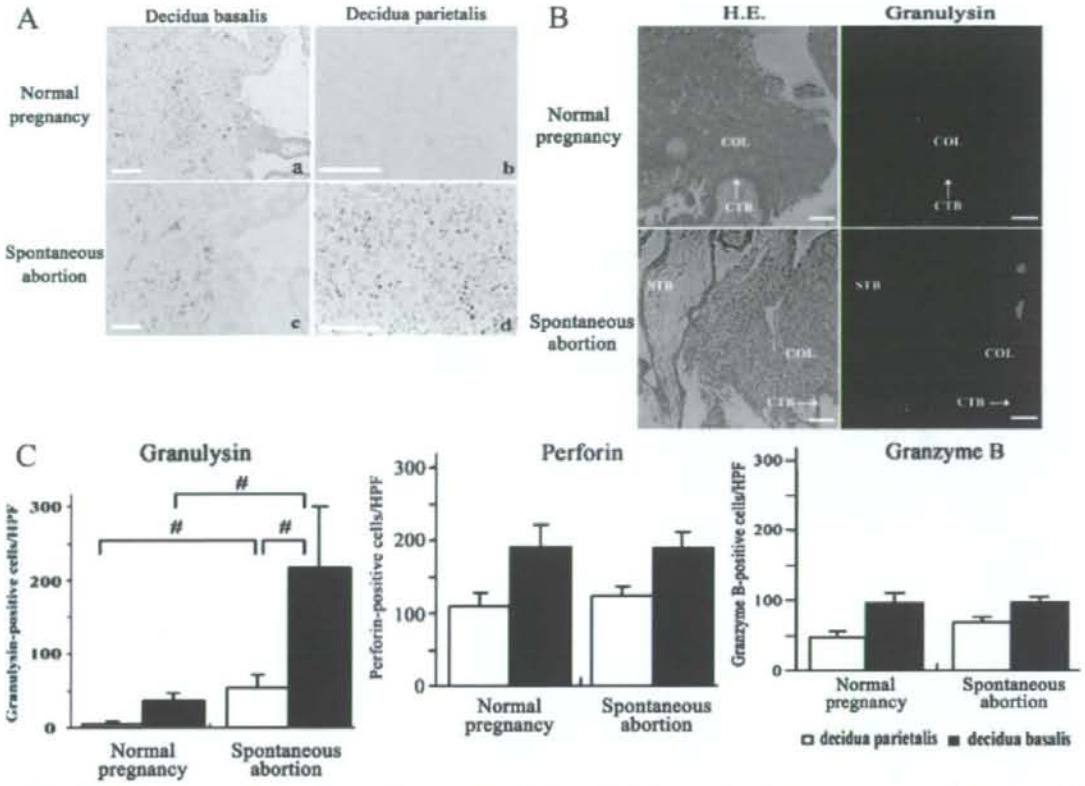

Figure 2. Expression of granule proteins in the decidua basalis and parietalis *in vivo*. **A:** Immunohistochemical studies showed expression of granulysin in the decidua basalis (**a**) and parietalis (**b**) in normal pregnancy, and decidua basalis (**c**) and parietalis (**d**) in spontaneous abortion at 8 weeks of gestation. Scale bars = 100 μm. **B:** Serial paraffin sections of the maternal-fetal interface were stained with H&E (left panels) and anti-granulysin (right panels) in normal pregnancy (upper panel) and spontaneous abortion (lower panels) of 8 weeks' gestation. Right panels were confocal microscopic images. Scale bars = 100 μm. **C:** Number of granulysin-positive cells (**left**), perforin-positive cells (**middle**), or granzyme B-positive cells (**right**). Two pairs of bars indicate the number of granule protein-positive cells in the decidua parietalis (white) and deciduas basalis (black) in normal pregnancy (left side, $n = 10$, gestational age 6–10 weeks) and in spontaneous abortion (right side, $n = 20$, gestational age 6–11 weeks) (#$P < 0.05$). Data are the means and standard deviations of 20 experiments. STB, syncytiotrophoblasts; CTB, cytotrophoblasts; COL, the proximal site of the cell column.

cytes from normal pregnancy or spontaneous abortion cases by flow cytometry. Granulysin expression was mainly detected in CD56^{bright} NK cells, but in only a few in CD3$^+$ T cells in spontaneous abortion cases (Figure 3B). The percentages of granulysin-positive cells in CD56^{bright} NK cells were 20.6 ± 1.3% and 33.5 ± 4.2% in normal pregnancy and spontaneous abortion, respectively ($P < 0.05$). The percentage of granulysin-positive CD56^{bright} NK cells in spontaneous abortion was significantly higher than that of normal pregnancy ($P < 0.05$), and that of CD56^{bright} NK cells were significantly higher than that of CD3 T cells both in normal pregnancy and spontaneous abortion ($P < 0.05$). There was, however, no difference in the percentage of granulysin-positive T cells between normal pregnancy and spontaneous abortion (Figure 3C). These results indicated that CD 56^{bright} NK cells, but not T cells, among decidual lymphocytes express granulysin in the decidua. Taken together, granulysin-positive CD 56^{bright} NK cells increased in the decidua but also accumulated at the deciduas basalis in spontaneous abortion cases.

Granulysin Staining Observed in Extravillous Trophoblasts of Spontaneous Abortion Cases

Several studies reported that apoptosis of EVTs was induced via activation of the tumor necrosis factor-α or Fas/FasL pathway in spontaneous abortion or preeclampsia.[1,21,22] In this context, the relationship between granulysin expression and apoptosis in EVTs was explored by immunohistochemistry. Interestingly, the expression of granulysin was detected in the nuclei of EVTs, which were reacted with cytokeratin antibodies, by diaminobenzidine staining (Figure 4A, a). The percentage of the granulysin-reactive cells was significantly increased in samples from spontaneous abortion compared with normal pregnancy (Figure 4B, 3.7 ± 0.9% versus 0.5 ± 0.5%, $P < 0.001$). To further examine the apoptosis of EVTs, we used two different methods: the terminal deoxynucleotidyl transferase dUTP nick-end labeling method and an antibody against cleaved cytokeratin-18, a detection marker in the early stage of apoptosis. Ter-

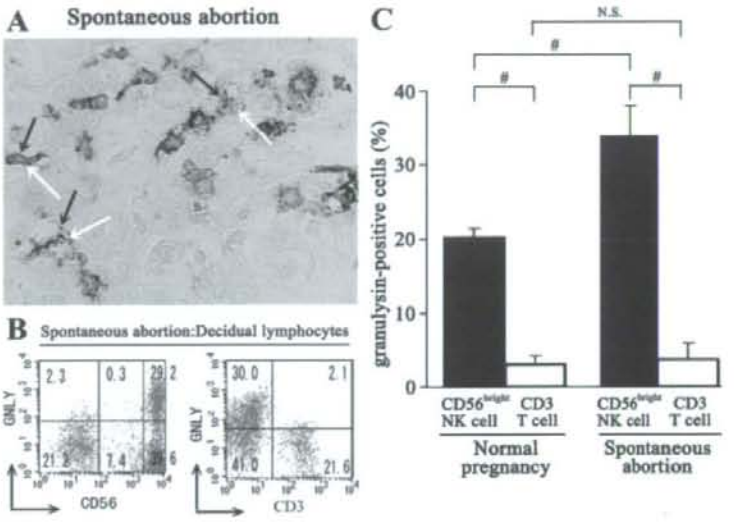

Figure 3. CD 56^{bright} NK cells expressed granulysin in spontaneous abortion *in vivo*. **A:** Immunostaining tissue section of decidua of spontaneous abortion at 8 weeks of gestation revealed that granulysin (**white arrows**, blue dots) was detected only in some CD56-positive cells (**black arrows**, brown). **B:** Granulysin (GNLY) expression in decidual lymphocytes from a spontaneous abortion case was assessed by fluorescence-activated cell sorting analyses on $CD56^{bright}$ cells or $CD3^+$ cells. Data are from one representative experiment of three performed. The intensity of fluorescein isothiocyanate staining (GNLY-positive) is shown on the *y* axis, while the intensity of CD56 (left panel) or CD3 (right panel) staining is plotted on the *x* axis. The numbers represent the percentages of the dots in each gated area. **C:** The percentages of granulysin-positive cells in $CD56^{bright}$ NK cells (black bars) and $CD3^+$ cells (white bars) in normal pregnancy (left panels) and spontaneous abortion (right panels) were shown ($*P < 0.05$). Data are the means and standard deviations of three independent experiments. N.S., not significant.

minal deoxynucleotidyl transferase dUTP nick-end labeling or cleaved cytokeratin-18 antibody reactive cells increased in spontaneous abortion cases, suggesting that these cells were dying due to apoptosis (Figure 4A, b and c). Furthermore, confocal studies clearly showed that some cytokeratin reactive EVTs invaded and detached from cytotrophoblast cell column in spontaneous abortion (Figure 4C, parts A and B). Granulysin expression was distinctly seen in both the cytoplasm and nuclei of these EVTs in spontaneous abortion (Figure 4C, parts C and D). On the other hand, granulysin expression was not detected in EVTs in normal pregnancy (Figure 4C, part E). Additionally, we next examined the granulysin mRNA expression in four choriocarcinoma cell lines, BeWo, JEG3 and JAR, and EVT cell line, HTR-8/SV40neo cells. These cells did not express granulysin mRNA (data not shown), suggesting that the granulysin in EVT cells was derived from the decidual NK cells. These results suggested that the increase in apoptotic EVT cells in spontaneous abortive tissue was related to the expression of granulysin in EVT cells by a mechanism in which uterine $CD56^{bright}$ NK cells transfer granulysin into EVTs.

Granulysin Produced by Uterine CD 56^{bright} NK Cells Accumulated into Nuclei of HTR-8/SV40neo Cells in vitro

We investigated the dynamic movement of native granulysin, which was derived from decidual lymphocytes, by an *in vitro* assay. In regard to this question, we checked the expression of granulysin on decidual lymphocytes obtained from normal pregnancy. Immunocytochemical staining showed that 1 ng/ml IL-2 enhanced granulysin expression in the cytoplasm of decidual lymphocytes after 24 hours of stimulation (Figure 5A). First, HTR-8/SV40neo cells were cultured with decidual lymphocytes with or without IL-2 stimulation. Spotted granulysin staining was found in HTR-8/SV40neo cells after 12 hours of coculture with IL-2-stimulated lymphocytes (Figure 5B). After an additional 6 hours, the expression level increased and the expression pattern was diffuse in the cytoplasm. Finally, 24 hours after coculture with decidual lymphocytes, a marked amount of granulysin staining was merged with nuclear staining (Figure 5C). On the other hand, granulysin staining was not detected in HTR-8/SV40neo with control. As shown in Figure 3B, CD 56^{bright} NK cells mainly possessed granulysin in decidual lymphocytes. After the isolation of CD 56-positive cells from IL-2-stimulated decidual lymphocytes, these isolated CD56-positive lymphocytes were cocultured with HTR-8/SV40neo cells. The percentages of CD56-positive cells were increased from 60% to 95% in the decidual lymphocytes after the isolation (data not shown). Confocal microscopic study also showed the similar results. As shown in Figure 5A, the expression of granulysin in decidual lymphocytes was detected only in the cytoplasm but not nuclei. At 24 hours after the culture, granulysin was colocalized at some large nuclei in HTR-8/SVneo cells, which were treated with CD56-positive lymphocytes with IL-2 (Figure 5D, arrows). On the other hand, granulysin was detected at around small nuclei but not in nuclei in the control sample, which was cocultured with CD56-positive lymphocytes without IL-2 stimulation (Figure 5D, arrowheads), indicating CD56-positive lymphocytes without IL-2 stimulation did not secrete granulysin. These results suggested that hyperactivated decidual CD56-positive NK cells secreted granulysin accumulated in nuclei of HTR-8/SVneo cells. Furthermore, separating the IL-2-stimulated decidual lymphocytes and HTR-8/SV40neo with a Transwell culture system completely abrogated the expression of granulysin in HTR-8/SV40neo, suggesting that cell-cell contact was required for granulysin transfer from decidual lymphocytes (Figure 5E). When decidual lymphocytes were treated with IL-2 con-

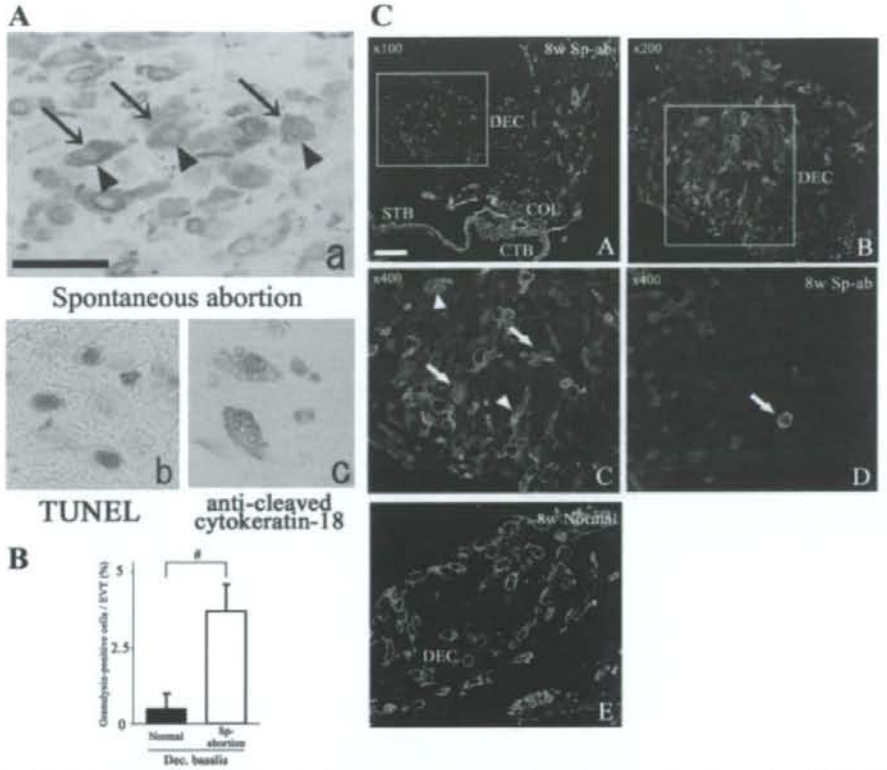

Figure 4. Extravillous trophoblast cells expressed granulysin in spontaneous abortion *in vivo*. **A:** Immunostaining tissue section of decidua of spontaneous abortion at 8 weeks of gestation revealed that granulysin (blue; **arrowheads**) was detected in cytokeratin-reactive cells (brown; **arrows**) in spontaneous abortion (**a**). Representative images of terminal deoxynucleotidyl transferase dUTP nick-end labeling staining (**b**) and anti-cleaved cytokeratin 18 staining (**c**) in spontaneous abortion case were shown. **B:** Quantitation of the granulysin-reactive cells in cytokeratin-reactive cells (EVTs) in the deciduas basalis from normal pregnancy (black, $n = 10$; gestational age, 6–10 weeks) and spontaneous abortion (white, $n = 20$; gestational age, 6–11 weeks). Cell counting was carried out on five randomized regions for each sample. Data are the means and standard deviations of 20 experiments ($\#P < 0.05$). **C:** Confocal microscopic images showed granulysin (red) and cytokeratin (green) staining in spontaneous abortion (**A–D**) and normal pregnancy (**E**) at 8 weeks of gestation. In **C**, expressions of granulysin were detected in the cytoplasm (**arrowheads**) and nuclei (**arrows**) in cytokeratin-reactive cells (green). **B** shows the region outlined by white line in **A**, and **C** is the region outlined in **B**. White bar, 100-μm scale bar; black bar, 50-μm scale bar. STB, syncytiotrophoblasts; CTB, cytotrophoblasts; COL, cell column; DEC, decidua basalis.

currently with concanamycin A, the inhibitor of perforin, before the coculture, concanamycin A treatment also blocked the expression of granulysin in HTR-8/SV40neo, suggesting that granulysin transfer is dependent on perforin expression (Figure 5F). These results suggested that CD 56^{bright} uNK cell-secreted native granulysin was transferred into the cytoplasm of EVTs in a cell-to-cell contact manner, resulting in the accumulation of granulysin into the nuclei of EVTs.

Apoptosis Is Induced by GFP-Fused Granulysin in Choriocarcinoma Cell Lines and HTR-8/SV40neo Cells

We next examined whether transfer of granulysin into nuclei correlates with apoptosis of EVTs by using a GFP-fused granulysin expression vector (Figure 1). As shown in Figure 6A, GFP-fused granulysin, as well as native granulysin, transferred from cytoplasm into nuclei in a time-dependent manner. The percentages of cells, of which granulysin was detected exclusively in nuclei, were 27.7 ± 2.9, 46.7 ± 1.89, and 89.7 ± 1.27 at 12, 24, and 48 hours, respectively, after transfection in HTR-8/SV40neo (Figure 6B). On the other hand, the percentage of GFP expression in control was 13.2 ± 2.02 at 12 hours and the level of GFP expression in the control was stable for at least 48 hours after transfection. Consequently, to characterize the granulysin-induced cell death, the cells were stained with Hoechst 33342 (nuclear staining) and an anti-cleaved cytokeratin 18 antibody (an apoptotic marker of the early stage with spotting) after granulysin transfection. Spotted staining was detected in granulysin-positive cells on anti-cleaved cytokeratin 18 panels (Figure 6C, upper panels), whereas diffused staining was in control-positive cells (Figure 6C, lower panels), respectively. Furthermore, condensed nuclei were partially detected at 48 hours after transfection in granulysin-positive cells, suggesting an increase in apoptosis (Figure 6D).

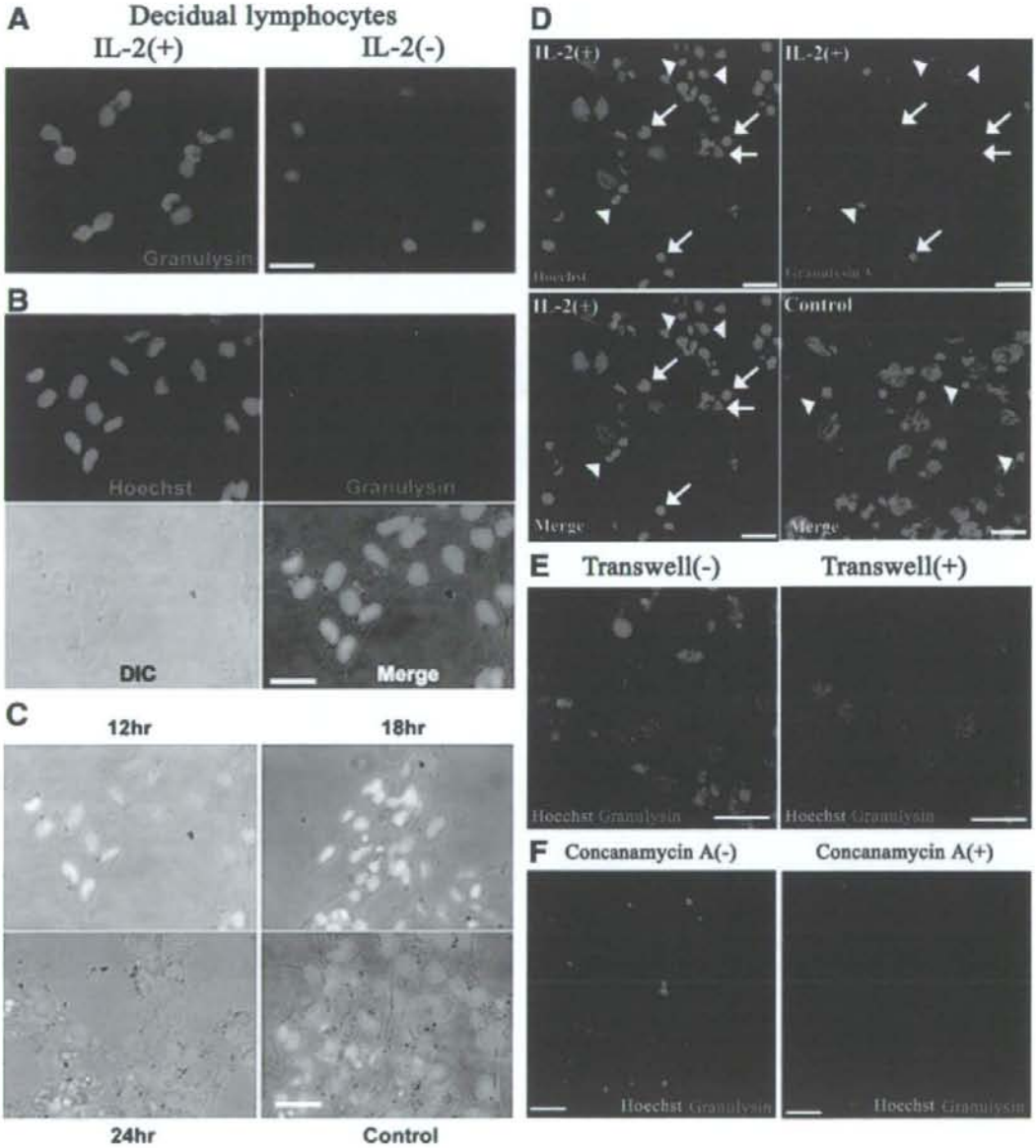

Figure 5. Localization of granulysin, derived from CD56$^+$ NK cells, in HTR-8/Svneo. Decidual lymphocytes, which were cultured with 1 ng/ml IL-2 for 24 hours, were cocultured with HTR-8/SV40neo for the indicated times. **A:** The reactivity of antibody against granulysin (red) on the decidual lymphocytes with IL-2 (left) for 24 hours was detected (blue: nuclear staining). Scale bar = 25 μm. **B:** HTR-8/SV40neo cells cocultured with decidual lymphocytes stimulated with IL-2 for 12 hours. Immunostaining of the samples showed that granulysin (red) was detected as dots in HTR-8/SV40neo. The right lower panel was the merged panel. Scale bar = 50 μm. **C:** These panels represented the localization of granulysin (red) in HTR-8/SV40neo at 12, 18, and 24 hours after coculture with IL-2-stimulated decidual lymphocytes. Scale bar = 50 μm. **D:** After the isolation of CD56-positive cells from IL-2-stimulated decidual lymphocytes, these CD56-positive cells were cocultured with HTR-8/SV40neo for 24 hours. Confocal microscopic images showed the colocalization (**white arrows**) of granulysin (red) and nuclear staining (blue) as well as the perinuclear localization (**arrowheads**) of granulysin. Control showed only the perinuclear localization (**arrowheads**) of granulysin. Scale bar = 25μm. **E:** HTR-8/SV40neo in the lower chamber was cocultured with IL-2-stimulated decidual lymphocytes, in the upper chamber with semipermeable Transwell membrane (right panel), or directly cocultured with IL-2-stimulated decidual lymphocytes (left panel) for 24 hours. Scale bar = 25 μm. **F:** HTR-8/SV40neo was directly cocultured with IL-2-stimulated decidual lymphocytes treated with (right panel) or without concanamycin A (left panel) for 24 hours. Both granulysin and nuclear staining are shown in red and blue, respectively. Scale bar = 25 μm.

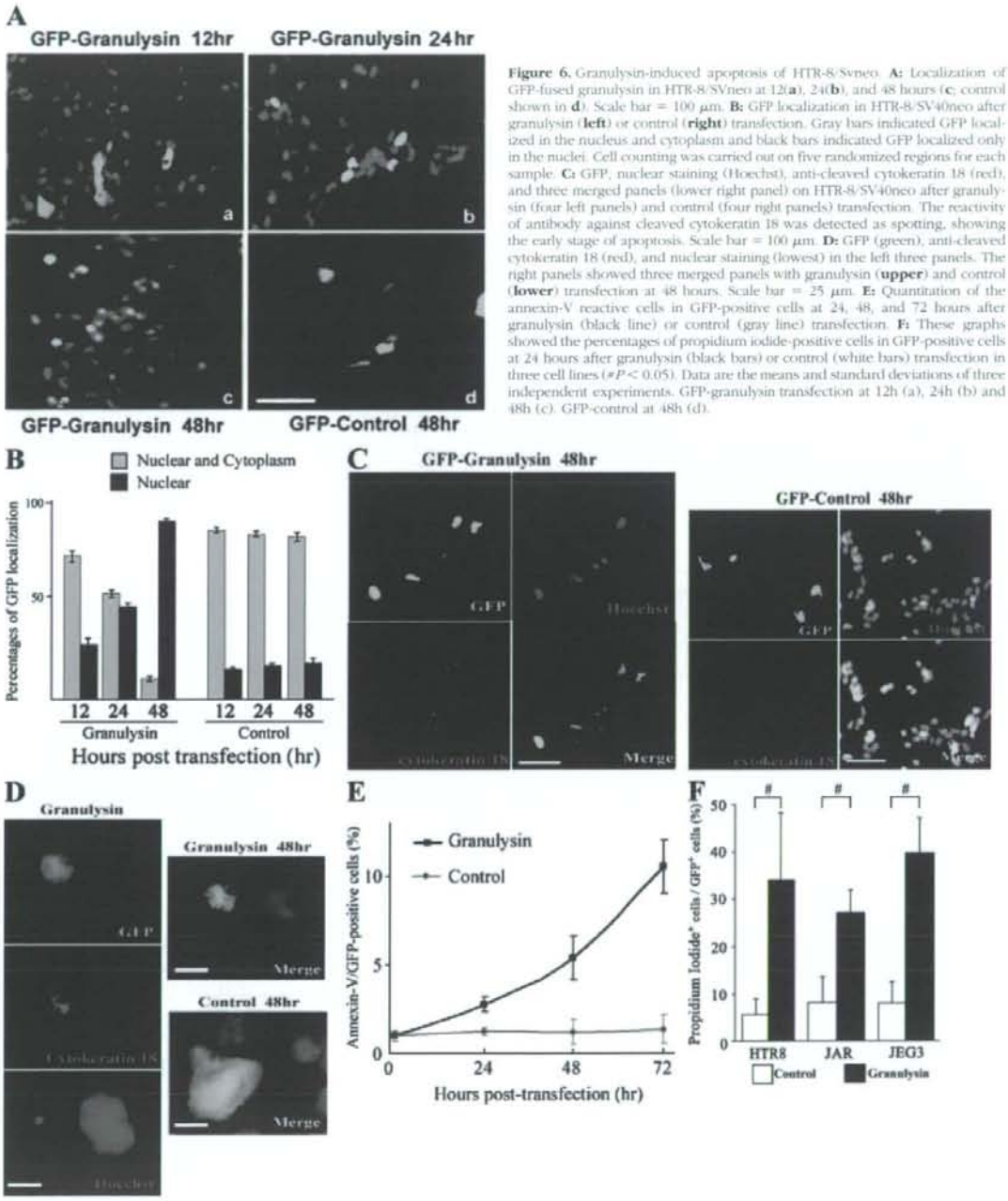

Figure 6. Granulysin-induced apoptosis of HTR-8/Svneo. **A:** Localization of GFP-fused granulysin in HTR-8/SVneo at 12(**a**), 24(**b**), and 48 hours (**c**; control shown in **d**). Scale bar = 100 μm. **B:** GFP localization in HTR-8/SV40neo after granulysin (**left**) or control (**right**) transfection. Gray bars indicated GFP localized in the nucleus and cytoplasm and black bars indicated GFP localized only in the nuclei. Cell counting was carried out on five randomized regions for each sample. **C:** GFP, nuclear staining (Hoechst), anti-cleaved cytokeratin 18 (red), and three merged panels (lower right panel) on HTR-8/SV40neo after granulysin (four left panels) and control (four right panels) transfection. The reactivity of antibody against cleaved cytokeratin 18 was detected as spotting, showing the early stage of apoptosis. Scale bar = 100 μm. **D:** GFP (green), anti-cleaved cytokeratin 18 (red), and nuclear staining (lowest) in the left three panels. The right panels showed three merged panels with granulysin (**upper**) and control (**lower**) transfection at 48 hours. Scale bar = 25 μm. **E:** Quantitation of the annexin-V reactive cells in GFP-positive cells at 24, 48, and 72 hours after granulysin (black line) or control (gray line) transfection. **F:** These graphs showed the percentages of propidium iodide-positive cells in GFP-positive cells at 24 hours after granulysin (black bars) or control (white bars) transfection in three cell lines (#$P < 0.05$). Data are the means and standard deviations of three independent experiments. GFP-granulysin transfection at 12h (a), 24h (b) and 48h (c). GFP-control at 48h (d).

Subsequently, the percentages of annexin V-positive cells among GFP-positive cells were estimated after granulysin transfection on HTR-8/SV40neo. The percentages of apoptotic cells were 2.7 ± 0.4, 5.7 ± 1.2, and 10.5 ± 1.5 at 24, 48, and 72 hours after transfection, respectively (Figure 6E). The kinetics of nuclear transport of granulysin coincided with the increase of apoptosis in EVTs after transfection. We further estimated the granulysin-induced cell death in choriocarcinoma cell lines, JEG3, and JAR cells as well as HTR-8/SV40neo, EVT cell line, by using propidium iodide. As shown in Figure 6F, cell death rates were significantly increased by granulysin transfection in all cell lines compared with control. Taken together, these results suggested that accumula-

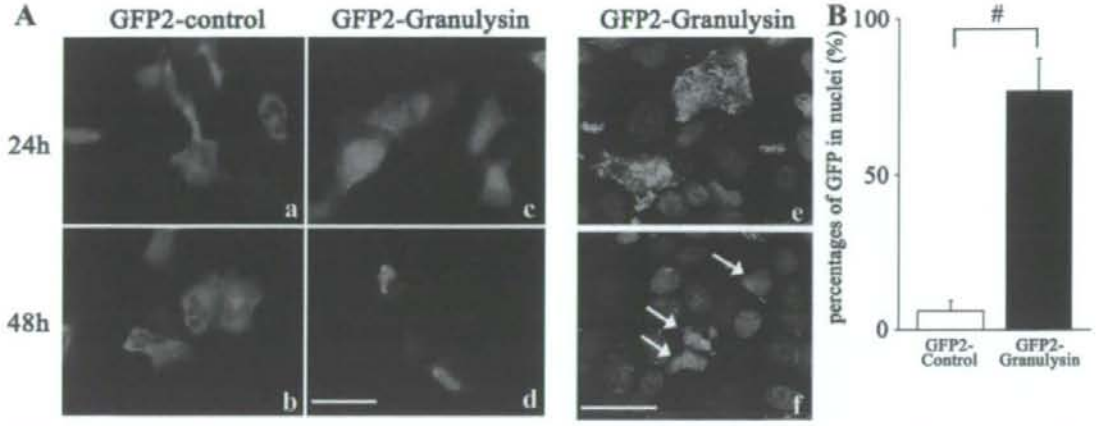

Figure 7. Nuclear accumulation of GFPx2-granulysin. **A:** Localization of the GFPx2-fused control (**a** and **b**) and granulysin (**c–f**) in HTR-8/SV40neo at 24 (**upper panels**) and 48 (**lower panels**) hours after transfection. These experiments were performed by both immunofluorescent microscopy (**a–d**) and confocal microscopy (**e** and **f**). Confocal microscopic images showed that GFPx2-fused granulysin (green, **arrows**) merged with Hoechst 33342 (blue) staining. GFPx2-control was detected only in peri-nuclei of HTR-8/SV40neo. Scale bar = 50 μm. **B:** GFP localization in HTR-8/SV40neo transfected with GFPx2-fused granulysin (black) or GFPx2-fused control (white) was evaluated. This graph showed the percentages of GFP localized only in the nuclei among GFP-positive cells. Cell counting was carried out on five randomized regions for each sample ($\#P < 0.05$). Data are the means and standard deviations of three independent experiments.

tion of granulysin into nuclei played important roles in the induction of apoptosis.

Active Accumulation of Granulysin into Nuclei on HTR8/SV40neo

The molecular weight of granulysin is 9 kd, and the weight of GFP-fused granulysin is 36 kd. It is therefore possible that both granulysin and GFP-fused granulysin easily pass through nuclear pores, because diffusion of substances into nuclei partially relies on the molecular weight, which is under 40 kd. To exclude the possibility of spontaneous nuclear diffusion of granulysin, we constructed a new vector, pEGFPx2-granulysin, which links granulysin to GFP-cDNA at the C′ end (Figure 1). Conceptually, neither GFPx2-granulysin nor the GFPx2-control, tandemly arranged GFP, should migrate into nuclei in a simple diffusion manner. An immunocytochemical fluorescent study showed that green dots were detected in cytoplasm and nuclei on GFPx2-granulysin transfected cells at 24 hours after transfection and then accumulated into nuclei at 48 hours after transfection (Figure 7A, c and d). Confocal microscopic studies clearly showed the nuclear accumulation of granulysin in HTR-8/SV40neo (Figure 7A, e and f). On the other hand, the GFPx2-control was only detected in cytoplasm, but not the nuclei, at 48 hours as well as 24 hours after transfection (Figure 7A, a and b). The percentage of cells, of which GFPx2-granulysin was detected exclusively in nuclei, was 76.9 ± 10.4, while that of GFPx2-control in nuclei was 5.9 ± 3.5 at 48 hours after transfection (Figure 7B), indicating that the percentage of GFPx2-granulysin in nuclei was significantly higher than that of GFPx2-control. These results indicated that granulysin actively migrated into nuclei of HTR-8/SV40neo independently of simple diffusion.

Granulysin-Induced Apoptosis Is Independent of Caspases

We finally explored the mechanism of granulysin-induced apoptosis by staining with propidium iodide, as a cell death marker, between granulysin and control transfection samples. As shown in Figure 6F, we obtained a significant difference in the percentages of propidium iodide-positive cells between granulysin and control transfection at 24 hours, and the percentages of propidium iodide-positive cells were 74.4 ± 4.7 and 18.3 ± 4.3 in granulysin and control at 48 hours after transfection in HTR-8/SV40neo (data not shown). Subsequently, we examined whether a general caspase inhibitor, z-VAD-FMK, inhibited granulysin-induced cell death in this experiment. No inhibition of cell death was observed on granulysin-transfected HTR-8/SV40neo cells, which were treated with z-VAD-FMK, after 48 hours. There were no effects on granulysin-induced cell death by treatment with the respective caspase inhibitors caspase-1, -3, -4, -6, -8, -9, -10 and -13 inhibitors, as well as a general caspase inhibitor (data not shown).

Discussion

In pregnancy, an increase in trophoblast apoptosis may induce insufficient trophoblast invasion and cause pregnancy-related disorders such as spontaneous abortion, preeclampsia, intrauterine growth restriction, or preterm labor.[1,2,21–24] On the other hand, numerous studies have reported that the predominance of Th2 over Th1 cytokines plays some roles in a successful pregnancy.[25,26] Olivares et al previously reported that decidual lymphocytes from human spontaneous abortion cases induced

apoptosis in JEG-3 cells, a choriocarcinoma cell line, by interacting with the target cells and IL-2-stimulated decidual lymphocytes.[1,2,21–24] Predominant Th1 type immunity is present in recurrent spontaneous abortion,[25,26] and apoptosis of trophoblasts is higher in spontaneous abortion with Th1 type immunity.[7] Furthermore, we also reported that serum granulysin is a good marker for detecting Th1 type immunity.[27] However, it is still unclear whether maternal lymphocytes can kill fetus-derived trophoblasts in spontaneous abortion cases.

This study has three major findings. The first is that the number of granulysin-positive $CD56^{bright}$ uNK cells was significantly higher in the decidua basalis in spontaneous abortion than in normal pregnancy, while there was no difference in the numbers of perforin-positive and granzyme B-positive cells. The number of granulysin-positive cells was also increased in decidual lymphocytes from spontaneous abortion cases than normal pregnancy subjects. Taken together, these findings showed that granulysin-positive $CD56^{bright}$ uNK cells were increased and accumulated in the decidua basalis in spontaneous abortion, suggesting that granulysin may be a key substance for spontaneous abortion. The second finding is that apoptosis of EVTs correlated with the granulysin transfer from $CD56^{bright}$ uNK cells in spontaneous abortion cases in both *in vivo* and *in vitro* experiments. Finally, granulysin transfer is dependent on both perforin and cell-cell contact, and transferred granulysin actively accumulated into nuclei in EVT cell line. Considering all results, we speculate that the mechanism by which granulysin induces apoptosis of EVTs plays an important role in inducing spontaneous abortion. This is the first report that granulysin, which is produced by $CD56^{bright}$ uNK cells, is involved in the induction of apoptosis of EVTs in spontaneous abortion by *in vivo* and *in vitro* experiments.

Numerous reports have shown that $CD8^+$ cytotoxic T cells and NK cells kill target cells such as virus-infected cells, some pathogenic microorganisms, tumor cells and other host cells, to defend the host against granulysin.[10,28] Although it is still an unknown mechanism by which decidual lymphocytes induce apoptosis on EVTs, we gave a new insight that granulysin, a cytotoxic granule protein of NK cells, contributes to the apoptosis of EVT in spontaneous abortion cases. Our previous report showed that granulysin is associated with the development of preeclampsia as a Th1 marker; this study showed that granulysin plays important roles affecting the development of a disease, spontaneous abortion, as well as defending the host.[29]

Our system, using expression vectors, gave new findings on the dynamic state of granulysin within cells. We have already reported that GFP-granulysin induces the cell death with nuclear accumulation in HeLa cells independently of caspase.[19] In this study, GFPx2-granulysin, which existed diffusely in the cytoplasm, gradually accumulated into nuclei, whereas GFPx2-control stabilized in the cytoplasm, suggesting that granulysin actively accumulates in the nuclei. Additionally, we showed that granulysin may be able to induce apoptosis in EVTs. As the analysis of molecular sequences demonstrated that granulysin has no nuclear localization signal among the well-known nuclear localization signals, the mechanism of granulysin accumulation in nuclei is unknown. To date, some reports have shown that several factors such as sphingomyelinase, intracellular calcium concentration, cytochrome *c* release, and apoptosis-inducing factor, played important roles in granulysin-induced apoptosis by using other systems, through which granulysin permeated to the target cells using a medium containing recombinant granulysin.[16,30–32] To resolve the mechanism by which granulysin induces apoptosis of EVTs, we checked the correlation between apoptosis-related molecules and granulysin expression. Some caspase inhibitors had no effect on granulysin-induced cell death. Consequently, we hypothesized that mitochondria, which is attacked by granulysin, releases apoptosis-inducing factor to cytoplasm, and then apoptosis-inducing factor translocates to the nuclei of EVTs. However, Western blotting and confocal microscopic studies showed that the translocation of apoptosis-inducing factor did not change together with granulysin. Thus, uNK-derived granulysin may induce apoptosis of EVTs by itself. There are some possibilities that direct or indirect chromatin binding of granulysin may affect chromatin remodeling, resulting in the induction of apoptosis, but this is still unknown. Therefore, further studies are needed to verify this mechanism.

Straszewski-Chavez et al also showed down-regulation of X-linked inhibition of apoptosis (XIAP) renders first-trimester trophoblast cells sensitive to Fas-mediated apoptosis.[33] In this study, granulysin expression did not affect the expression of XIAP on HTR-8/SV40neo cells (data not shown). The decrease in XIAP induced the activation of caspase-8, -9 and -3,[33] but granulysin-induced apoptosis was independent of caspases in this study and a previous study.[19] EVTs inhibit caspase cascade activation by XIAP, because first trimester trophoblast cells express both Fas and FasL. In other words, XIAP may be an indispensable factor for physiological function, such as differentiation or invasion of trophoblasts.[33] Given that granulysin has an etiological factor, but not a physiological factor, it may effectively induce apoptosis in EVTs independently of caspases and XIAP.

Olivares et al and Kokawa et al have suggested that excessive destruction of trophoblasts by apoptosis results in spontaneous abortion.[2,21] In regard to this point, several apoptosis pathways of EVT are known. First, Reister et al reported that macrophages secrete tumor necrosis factor-α and tumor necrosis factor-α induces apoptosis of EVT.[23] Second, Mor et al showed that isolated first-trimester trophoblast cells can express Fas on their surface and the Fas ligand could induce apoptotic death of trophoblasts.[33] Considering all our results *in vivo* and *in vitro*, we propose a third pathway, the granulysin pathway, in the course of apoptosis of EVT by uNK cells.

Acknowledgments

We thank Dr. Naoko Imamoto, RIKEN, Japan, for providing the pGFPx2 expression vector and Dr. Graham, Queen's University, Canada, for providing HTR-8/SV40neo. We also thank Dr. Yoshihiro Yoneda, Depart-

ment of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, for helpful and kind discussions.

References

1. Kaufmann P, Black S, Huppertz B: Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 2003, 69:1–7
2. Olivares EG, Munoz R, Tejerizo G, Montes MJ, Gomez-Molina F, Abadia-Molina AC: Decidual lymphocytes of human spontaneous abortions induce apoptosis but not necrosis in JEG-3 extravillous trophoblast cells. Biol Reprod 2002, 67:1211–1217
3. Namba Y, Hondo E, Morimoto M, Nakamura O, Kusakabe K, Ito M, Saito S, Sagara E, Kiso Y: A study of reproductive performance in pregnant IL-2 receptor beta-chain overexpressed transgenic mice. J Vet Med Sci 2001, 63:99–101
4. Nishikawa K, Saito S, Morii T, Hamada K, Ako H, Narita N, Ichijo M, Kurahayashi M, Sugamura K: Accumulation of CD16-CD56+ natural killer cells with high affinity interleukin 2 receptors in human early pregnancy decidua. Int Immunol 1991, 3:743–750
5. Moffett-King A: Natural killer cells and pregnancy. Nat Rev Immunol 2002, 2:656–663
6. Dosiou C, Giudice LC: Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. Endocr Rev 2005, 26:44–62
7. Lee J, Choi BC, Cho C, Hill JA, Baek KH, Kim JW: Trophoblast apoptosis is increased in women with evidence of TH1 immunity. Fertil Steril 2005, 83:1047–1049
8. Zheng LM, Ojcius DM, Young JD: Perforin-expressing cells during spontaneous abortion. Biol Reprod 1993, 48:1014–1019
9. Yamada H, Shimada S, Morikawa M, Iwabuchi K, Kishi R, Onoe K, Minakami H: Divergence of natural killer cell receptor and related molecule in the decidua from sporadic miscarriage with normal chromosome karyotype. Mol Hum Reprod 2005, 11:451–457
10. Pena SV, Hanson DA, Carr BA, Goralski TJ, Krensky AM: Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. J Immunol 1997, 158:2680–2688
11. Stenger S, Rosat JP, Bloom BR, Krensky AM, Modlin RL: Granulysin: a lethal weapon of cytolytic T cells. Immunol Today 1999, 20:390–394
12. Clayberger C, Krensky AM: Granulysin. Curr Opin Immunol 2003, 15:560–565
13. Hanson DA, Kaspar AA, Poulain FR, Krensky AM: Biosynthesis of granulysin, a novel cytolytic molecule. Mol Immunol 1999, 36: 413–422
14. Sarwal MM, Jani A, Chang S, Huie P, Wang Z, Salvatierra O, Jr., Clayberger C, Sibley R, Krensky AM, Pavlakis M: Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. Hum Immunol 2001, 62:21–31
15. Wang Z, Choice E, Kaspar A, Hanson D, Okada S, Lyu SC, Krensky AM, Clayberger C: Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin. J Immunol 2000, 165:1486–1490
16. Kaspar AA, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A, Hanson DA, Kluck RM, Hitoshi Y, Johnson DE, Froelich CJ, Thompson CB, Newmeyer DD, Anel A, Clayberger C, Krensky AM: A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol 2001, 167:350–356
17. Shresta S, Heusel JW, Macivor DM, Wesselschmidt RL, Russell JH, Ley TJ: Granzyme B plays a critical role in cytotoxic lymphocyte-induced apoptosis. Immunol Rev 1995, 146:211–221
18. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H: Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol 1996, 14:207–232
19. Takamori Y, Ogawa K, Nagata K, Takano S, Nakamura M: Granulysin induces cell death with nuclear accumulation. J Med Dent Sci 2005, 52:1–7
20. Ogawa K, Takamori Y, Suzuki K, Nagasawa M, Takano S, Kasahara Y, Nakamura Y, Kondo S, Sugamura K, Nakamura M, Nagata K: Granulysin in human serum as a marker of cell-mediated immunity. Eur J Immunol 2003, 33:1925–1933
21. Kokawa K, Shikone T, Nakano R: Apoptosis in human chorionic villi and decidua during normal embryonic development and spontaneous abortion in the first trimester. Placenta 1998, 19:21–26
22. Halperin R, Peller S, Rotschild M, Bukovsky I, Schneider D: Placental apoptosis in normal and abnormal pregnancies. Gynecol Obstet Invest 2000, 50:84–87
23. Reister F, Frank HG, Kingdom JC, Heyl W, Kaufmann P, Rath W, Huppertz B: Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. Lab Invest 2001, 81:1143–1152
24. DiFederico E, Genbacev O, Fisher SJ: Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. Am J Pathol 1999, 155:293–301
25. Raghupathy R: Th1-type immunity is incompatible with successful pregnancy. Immunol Today 1997, 18:478–482
26. Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S: Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat Med 1998, 4:1020–1024
27. Sakai M, Ogawa K, Shiozaki A, Yoneda S, Sasaki Y, Nagata K, Saito S: Serum granulysin is a marker for Th1 type immunity in pre-eclampsia. Clin Exp Immunol 2004, 136:114–119
28. Ma LL, Spurrell JC, Wang JF, Neely GG, Epelman S, Krensky AM, Mody CH: CD8 T cell-mediated killing of Cryptococcus neoformans requires granulysin and is dependent on CD4 T cells and IL-15. J Immunol 2002, 169:5787–5795
29. Qiu C, Saito S, Sakai M, Ogawa K, Nagata K, Williams MA: Plasma granulysin concentrations and preeclampsia risk. Clin Biochem 2006, 39:1016–1021
30. Gamen S, Hanson DA, Kaspar A, Naval J, Krensky AM, Anel A: Granulysin-induced apoptosis. I. Involvement of at least two distinct pathways J Immunol 1998, 161:1758–1764
31. Pardo J, Perez-Galan P, Gamen S, Marzo I, Monleon I, Kaspar AA, Susin SA, Kroemer G, Krensky AM, Naval J, Anel A: A role of the mitochondrial apoptosis-inducing factor in granulysin-induced apoptosis. J Immunol 2001, 167:1222–1229
32. Okada S, Li Q, Whitin JC, Clayberger C, Krensky AM: Intracellular mediators of granulysin-induced cell death. J Immunol 2003, 171:2556–2562
33. Straszewski-Chavez SL, Abrahams VM, Funai EF, Mor G: X-linked inhibitor of apoptosis (XIAP) confers human trophoblast cell resistance to Fas-mediated apoptosis. Mol Hum Reprod 2004, 10:33–41

Human Reproduction Vol.23, No.9 pp. 2080–2085, 2008
Advance Access publication on June 25, 2008
doi:10.1093/humrep/den187

Erythrocytes counteract the negative effects of female ageing on mouse preimplantation embryo development and blastocyst formation

Rie Fukuhara[1], Shunsaku Fujii, Rika Nakamura, Ei Yuzawa, Hidetaka Kimura, Atsushi Fukui and Hideki Mizunuma

Department of Obstetrics and Gynecology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

[1]Correspondence address. E-mail: fukuhara@cc.hirosaki-u.ac.jp

BACKGROUND: The low developmental competence of embryos from ageing females remains an enigma; it is presumably attributable to oxidative stress. A number of antioxidant mechanisms exist in the erythrocyte and these have been investigated in other cells and tissues. However, very few studies have reported the effects of erythrocyte supplementation on developmental competence in ageing embryos. METHODS: In Experiment 1, IVF embryos from young (7–10 weeks) mice were cultured in medium supplemented with an oxidizing agent, hypoxanthine/xanthine oxidase, in the presence and absence of erythrocytes. In Experiment 2, the development of embryos derived from young and ageing (40–50 weeks) female mice was assessed in the presence and absence of erythrocytes. RESULTS: In Experiment 1, the presence of hypoxanthine/xanthine oxidase significantly inhibited embryo development ($P < 0.0001$). Erythrocyte supplementation clearly overcame the detrimental effects in a dose-related manner. In Experiment 2, in the absence of erythrocytes, developmental competence was significantly lower in embryos from ageing females than in those from young females ($P < 0.01$). However, in ageing females, the supplementation of erythrocytes significantly promoted the development of embryos to the blastocyst stage (51.1% versus 77.3%; $P < 0.01$). CONCLUSIONS: Supplementation with erythrocytes can counteract the negative effect of maternal ageing on embryo development and blastocyst formation.

Keywords: ageing; antioxidant; embryo; erythrocyte; reactive oxygen species

Introduction

Female fertility declines with age. A variety of factors may contribute to this age-related decline in fertility, including reduced numbers of oocytes, poor oocyte quality and diminished endometrial receptivity. However, one of the well-recognized aspects of reproductive decline is poor oocyte quality, as seen in the significantly better pregnancy rates during ovum donation (Navot *et al.*, 1991). Aneuploidy (Koehler *et al.*, 1996), mitochondrial DNA mutations (Keefe *et al.*, 1995; Wei *et al.*, 1998), mitochondrial dysfunction (Van Blerkom *et al.*, 1995) and cytoskeletal alterations (Battaglia *et al.*, 1996) have been suggested to play a part in the age-associated reduction of oocyte quality (Baird *et al.*, 2005). In addition, reduction in rates of blastocyst formation with increasing age has been reported (Janny and Menezo, 1996; Keefe, 1997). Although many hypotheses have been suggested to account for the relationship between female age and oocyte quality, the mechanisms that underlie these changes remain unclear.

The free radical theory of ageing emphasizes that biomolecules are attacked by free radicals and that the repeated damage to biomolecules plays a major role in ageing (Harman, 1956, 1981). It is suggested that reactive oxygen species (ROS) play essential roles in the age-related decline in female fertility (Tarin, 1995, 1996). ROS may originate either directly from oocytes and embryos or from their surroundings, and are detrimental to embryo development (Goto *et al.*, 1993). Oxidative damage may result from overproduction and/or decreased clearance of ROS by the scavenging mechanisms. Oxidative stress experimentally induced by oxidizing agents (Liu and Keefe, 2000; Liu *et al.*, 1999, 2000), visible light or high atmospheric O_2 concentrations (Kitagawa *et al.*, 2004) has deleterious effects on the survival and nuclear and cytoplasmic maturation of oocytes and the developmental competence of the embryo. On the other hand, multiple defence mechanisms are present in both embryos and their surroundings (Guerin *et al.*, 2001; Salmen *et al.*, 2005). ROS scavengers counteract the disturbing effects of female ageing on oocyte

quality and quantity in the mouse (Tarin *et al.*, 2002). In embryos from ageing females, antioxidant defence mechanisms may be insufficient and oxidative stress may increase. Therefore, it is particularly important to pay careful attention to culture conditions for embryos from ageing females to protect them from oxidative stress *in vitro*.

The established function of the erythrocyte is to transport oxygen to tissues and remove carbon dioxide, because of which the erythrocytes come into contact with highly toxic oxygen species during this process. Against this, a number of antioxidant mechanisms exist in the erythrocyte (Richards *et al.*, 1998a,b). The protective effect of erythrocytes against ROS has been discussed with respect to other cells and tissues subjected to oxidative stress (Richards *et al.*, 1998a,b). On the other hand, it has been reported (Matsuoka *et al.*, 1995) that, in mice, small numbers of erythrocytes effectively protect cultured oocytes and embryos from oxidative stress and that the presence of erythrocytes improves the early development of embryos by their antioxidant effect. However, very few studies have reported the effects of erythrocyte supplementation on developmental competence of oocytes and embryos *in vitro*.

The objectives of this study were to evaluate the adverse effects of exogenously induced ROS by investigating the effect of hypoxanthine/xanthine oxidase on mouse embryo development *in vitro*, to examine the protective effect of erythrocyte supplementation and then to examine the effects of erythrocytes on the blastocyst development rate and the hatching rate of embryos derived from ageing female mice.

Materials and Methods

Animals

Institute of Cancer Research (ICR) mice were originally purchased from central laboratories for experimental animals Japan, Inc. and subsequently bred in our laboratory. The mice were maintained on a 12 h light:12 h dark photoperiod in a temperature-controlled room at 21–23°C. All experiments were performed in accordance with the guidelines for animal experimentation of Hirosaki University. ICR mice were used at 7–10 weeks of age as young mice, and at 40–50 weeks of age as ageing mice.

Chemicals and culture media

All chemicals used in the study were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise stated.

The basic medium used for the culture of embryos was Quinn's Advantage Protein Plus (QA-P+) system (Sage, Oxford, UK), and QA-P+Fertilization medium for fertilization procedures. The original formulation of QA-P+Fertilization and cleavage medium was modified by the addition of citrate, non-essential amino acids, taurine and calcium lactate. The original formulation of the QA-P+Blastocyst medium was modified by the addition of non-essential and essential amino acids, taurine, glutathione, minimum essential medium, vitamins and calcium lactate. Embryos were cultured to the blastocyst stage in QA-P+sequential media. Embryos were cultured in each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 800 μl/well of the culture medium, which was covered with mineral oil in a humidified atmosphere of 5% CO_2 at 37°C.

Oocyte isolation, IVF and in vitro culture

Superovulation was induced in the female mice by i.p. injection of 5 IU pregnant mare serum gonadotrophin (Serotropin, Teikokuzouki, Tokyo, Japan) followed 48 h later by an i.p. injection of 5 IU HCG (Mochida, Tokyo, Japan). The mice were sacrificed by cervical dislocation 14 h after HCG injection and ovulated oocytes were retrieved from the oviducts. Spermatozoa from the cauda epididymis from mature ICR male mice (aged 10–12 weeks) were collected and capacitated in QA-P+Fertilization medium for 1 h at 37°C. Oocytes were then inseminated *in vitro* with 1.0×10^6/ml capacitated spermatozoa in medium. Five hours after insemination, oocytes were denuded of surrounding cumulus cells by repeated pipetting and then washed several times in culture medium.

The normal, fertilized embryos were then transferred to 4-well multidishes for culture. Culture was performed in 800 μl of QA-P+ system medium, covered with mineral oil, in a humidified atmosphere of 5% CO_2 at 37°C for 5 days. Medium was equilibrated in this atmosphere for at least 3 h before culture. The numbers of embryos that developed to the 2-cell, 4-cell, 8-cell, morula, blastocyst, hatching and hatched blastocyst stages were determined under an inverted microscope (SZX12; Olympus, Tokyo, Japan).

Preparation of erythrocytes

Blood was collected (1.5 ml from each mouse) from ICR male mice, and we used two mice for each set of experiments. Citrate–phosphate–dextrose solution was used as an anticoagulant. Whole blood was filtered through a Sepacell RN-20 leukocyte depletion filter (Asahi Medical Co., Tokyo, Japan), followed by centrifugation at 3000*g* for 10 min and the plasma and buffy coat was removed. Erythrocytes were washed three times by centrifugation at 3000 g for 10 min using ice-cold Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS). The concentration of erythrocytes was counted using an improved Neubauer haemocytometer, and the erythrocytes were resuspended in culture medium for the following experiments.

Total cell number of blastocysts

On Day 5, blastocysts were collected, fixed with 4% (w/w) paraformaldehyde in PBS and stained with Hoechst 33342 (Dojindo, Tokyo, Japan). The blastocysts were then washed three times with PBS and put on a glass slide with a coverslip. The total number of nuclei were counted under a fluorescence microscope (Axiovert 200M; Carl Zeiss Inc., Jena, Germany) with a UV filter.

Experiment 1

To investigate the effect of ROS on mouse embryos, embryos obtained from young mice were cultured in medium containing 0.5 mM hypoxanthine and 0.01 U/ml xanthine oxidase. The cleavage rate and the rate of development to the blastocyst stage were examined. The effect of erythrocytes on the *in vitro* development of IVF embryos was examined at haematocrit values of 0.001%, 0.01%, 0.1% and 1% (5×10^4, 5×10^5, 5×10^6 and 5×10^7 erythrocytes/ml): the numbers of mice were 23, 5, 5, 5, 10 and 8 in each group, respectively, and the total numbers of embryos were 532, 153, 171, 165, 400 and 283 in each group.

Experiment 2

To examine the effect of erythrocytes on IVF embryos from ageing mice, development rates were compared between embryos from young and ageing mice in the presence of erythrocytes (haematocrit 0.2%; 1×10^7 erythrocytes/ml) during *in vitro* culture. The numbers of mice used were 23, 25, 39 and 34 (young control, young supplemented erythrocytes, old control and old supplemented

erythrocytes), and the total numbers of oocytes and embryos were 532, 607, 485 and 480, and 477, 542, 390 and 386 in each group, respectively.

Statistical analysis

Cleavage rate, development rate to the blastocyst stage, hatching rate and cell counts were evaluated as percentages or cell numbers and presented as mean (SEM). For statistical analysis, percentages were subjected to angular transformation. The data were analysed by one-way analysis of variance and subjected to Fisher's protected least-significant difference analysis using StatView software (SAS Institute, Inc., Cary, NC, USA). Differences among groups were considered significant when the *P*-value was <0.05.

Results

Experiment 1

Effects of hypoxanthine/xanthine oxidase on the development of IVF embryos

The presence of hypoxanthine/xanthine oxidase in the culture medium significantly decreased the cleavage rates of embryos compared with control (98% versus 44%, $P < 0.0001$). Although most embryos cultured in control medium developed to the blastocyst stage (82%), embryos exposed to hypoxanthine/xanthine oxidase were completely arrested at the 1- or 2-cell stage, suggesting that hypoxanthine/xanthine oxidase elicits potent inhibition of the development of embryos (Table I, Fig. 1).

Effects of erythrocytes on development of IVF embryos cultured with hypoxanthine/xanthine oxidase

The addition of erythrocytes to medium supplemented with hypoxanthine/xanthine oxidase markedly improved preimplantation development (Table I). The percentages of embryos that developed to the 2-cell, 4-cell, morula and blastocyst stages increased significantly ($P < 0.05$) in an erythrocyte concentration-related manner (Table I, Fig. 1).

Experiment 2

Effects of female ageing on development of IVF embryos

No significant differences between young and ageing control groups were detected in the rates of development of IVF embryos to the 2-cell stage (Table II). However, the percentages of embryos that reached the 4-cell, morula, blastocyst and hatching blastocyst formation were significantly lower in the ageing control group than in the young control group (4-cell stage, 94.7% versus 84.7%, $P = 0.01$; morula, 88.0% versus 71.0%, $P = 0.001$; blastocyst, 82.0% versus 51.1%, $P < 0.0001$; hatching blastocyst, 49.9% versus 30.4%, $P = 0.0007$). The average cell number at the blastocyst stage in the ageing control group was significantly lower than that in the young control group (63.9 versus 84.9; $P < 0.01$), strongly indicating that female ageing is associated with deterioration in oocyte quality.

Effects of erythrocytes on development of IVF embryos from young and ageing mice

In embryos from young mice, no significant differences were observed in the rate of development to the 2-cell, 4-cell, morula, blastocyst and hatching blastocyst stages between the erythrocyte-supplemented group and the control. The total number of cells in blastocysts derived from IVF embryos cultured in medium with erythrocytes was lower than that in control blastocysts (73.0 versus 84.9; $P = 0.0003$).

In ageing mice, on the other hand, the addition of erythrocytes promoted the development of embryos to the 4-cell stage (84.7% for control versus 91.9%; $P = 0.03$), morula stage (71.0% versus 84.2%; $P = 0.006$), blastocyst stage (51.1% versus 77.3%; $P < 0.0001$) and hatching blastocyst stage (30.4% versus 44.4%; $P = 0.002$). For ageing mice, the development rates of embryos cultured with erythrocytes improved to a similar extent as those of embryos from young mice, suggesting that erythrocytes counteracted the negative

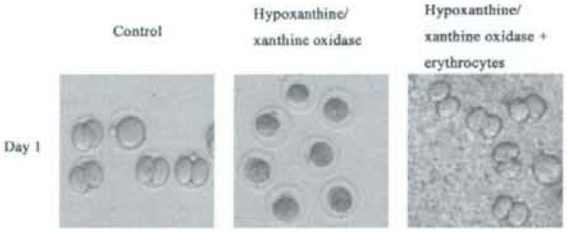

Figure 1: Effects of hypoxanthine/xanthine oxidase and erythrocytes on development of mouse IVF embryos.
In control culture, an average of 98% embryos cleaved at Day 2, and 82% developed to blastocysts. Addition of hypoxanthine/xanthine oxidase inhibited cleavage and development, causing developmental arrest and cell death. Addition of erythrocytes improved the embryonic development.

Table I. Effects of hypoxanthine/xanthine oxidase and erythrocytes supplementation on embryo development.

Supplements	No. of IVF embryos	No. of mice	Mean percentage (=SE) developed stage of IVF embryos			
			2-cell	4-cell	Morula	Blastocyst
Control	477	23 98.2 (0.7)[a]	94.7 (1.1)[a]	88.0 (1.7)[a]	82.0 (1.7)[a]	
HX/XOD	142	5	44.0 (15.0)[b]	0[b]	0[b]	0[b]
HX/XOD+Erythrocyte 0.001	150	5	51.8 (4.4)[b]	0[b]	0[b]	0[b]
HX/XOD+Erythrocyte 0.01	135	5	62.7 (12.0)[b]	11.0 (7.8)[b]	0[b]	0[b]
HX/XOD+Erythrocyte 0.1	331	10	90.8 (3.0)[c]	64.7 (8.5)[c]	31.5 (6.8)[c]	8.2 (1.4)[b]
HX/XOD+Erythrocyte 1	241	8	95.0 (1.7)[a,c]	88.7 (3.6)[a]	75.5 (9.7)[a]	10.1 (2.4)[c]

[a,b,c]Different superscripts within each column are significantly different ($P < 0.05$).
HX/XOD = 0.5 mM hypoxanthine + 0.01 U/ml xanthine oxidase; SE, standard error; IVF, *in vitro* fertilization.

Table II. Effects of female ageing and addition of erythrocytes to culture medium on the development of mouse IVF embryos.

Mouse age	Culture condition	No. of IVF embryos	No. of mice	Mean percentage (±SE) of IVF embryos developed to				
				2-cell	4-cell	Morula	Blastocyst	Hatching blastocyst
Young	Control	477	23	98.2 (0.7)	94.7 (1.1)^a	88.0 (1.7)^a	82.0 (1.7)^a	49.9 (3.5)^a
	+Erythrocyte	542	25	98.6 (0.6)	94.6 (1.4)^a	87.6 (1.6)^a	77.6 (1.6)^a	43.8 (3.9)^a
Old	Control	390	39	96.6 (1.4)	84.7 (2.4)^b	71.0 (3.7)^b	51.1 (2.9)^b	30.4 (3.1)^b
	+Erythrocyte	386	34	96.8 (1.0)	91.9 (1.8)^a	84.2 (1.9)^a	77.3 (1.8)^a	44.4 (3.4)^a

^{a,b}Different superscripts in each parameter were significantly different ($P < 0.05$).
SE, standard error; IVF, *in vitro* fertilization.

effects of ageing on the development of embryos from ageing females. The total number of cells in blastocysts derived from IVF embryos and cultured in medium with erythrocytes was significantly higher compared with the control (63.9 versus 72.4; $P = 0.015$).

Discussion

The results of the present study demonstrate clearly that hypoxanthine/xanthine oxidase, a potent oxidizing agent, inhibits the development of mouse preimplantation embryos and that erythrocytes completely overcome the inhibitory effects of the oxidizing agent.

Early mammalian embryos are susceptible to damage caused by ROS. ROS, up to a certain concentration, play important roles in the normal development of oocytes and embryos; however, once the ROS level exceeds a certain level, they cause oxidative stress and become toxic. It has been shown that embryo development is retarded or even arrested by exposure to media with high oxygen tension (Kitagawa *et al.*, 2004). Under high oxygen tension, excessive ROS, such as hydrogen peroxide and superoxide radicals, are produced (Goto *et al.*, 1993) and these ROS are considered to react with extremely high rate constants with amino acids, phospholipids, nucleotides and organic acids (Orsi and Leese, 2001), and therefore cause serious damage to embryos (Fujitani *et al.*, 1997; Agarwal *et al.*, 2003). On the other hand, it has been shown that ROS are produced in cultured embryos exposed to atmospheric oxygen tension, and that addition of free radical scavengers can ameliorate the 2-cell block in mouse embryo culture (Legge and Sellens, 1991). Since then, many reports have appeared describing the search for appropriate agents that can overcome the detrimental effects of free radicals (Liu *et al.*, 1999). For example, several of these antioxidants, such as superoxide dismutase (SOD), catalase, cysteine and vitamin E (Olson and Seidel, 2000; Orsi and Leese, 2001; Wang *et al.*, 2002; Ali *et al.*, 2003), have been tested. Thus far, however, the effects of these chemicals have not been consistent, probably because of the difference in the animal models or culture medium composition; the exact reason remains unclear (Bavister, 1995; Orsi and Leese, 2001).

Erythrocytes, on the other hand, have an antioxidant mechanism in addition to the transport of oxygen. Therefore, it is of interest to study the effect of erythrocytes on embryo development *in vitro*. It is reported that erythrocytes alleviate post-ischaemic reperfusion injury in the liver (Motoyama *et al.*, 2000) and the heart (Nohl *et al.*, 1981), and that co-culture of human umbilical vein endothelial cells and erythrocytes removes the ROS load (Richards *et al.*, 1998a,b). Erythrocytes are not only easily available, but they are also superior to other antioxidant agents. This is because erythrocytes contain a group of scavenging enzymes, such as SOD, catalase and glutathione, as well as haemoglobin, which works as a nitric oxide scavenger and suppresses ROS generation. Indeed, it has been reported that the addition of haemoglobin improves embryo development to the blastocyst stage and significantly increases the number of cells per blastocyst (Park *et al.*, 2000, 2001; Kim *et al.*, 2006). Moreover, combined administration of SOD or catalase with haemoglobin has been reported to increase the survival and fertilization rates of cryopreserved mouse oocytes (Dinara *et al.*, 2001), strongly supporting the view that erythrocytes are the most appropriate agents for regulating ROS levels. In fact, as shown in Table I, we found that erythrocyte supplementation clearly overcame the detrimental effects of hypoxanthine/xanthine oxidase in a concentration-related manner. It has been reported that the addition of erythrocytes to the culture medium overcomes the 2-cell block in mouse IVF embryos and improves embryo development (Matsuoka *et al.*, 1995; Musoh *et al.*, 2002). The results of the present study support these data.

The result of the present study, on the other hand, demonstrates that erythrocyte supplementation improved the development of embryos from aged mice. It has been well documented that developmental competence is low in embryos from ageing individuals compared with those from young individuals (Janny and Menezo, 1996). Oocytes from young females and embryos cultured with an oxidizing agent show cytoskeletal and chromosomal aberrations (Tarin, 1996) and growth arrest (Liu *et al.*, 1999). These morphological changes are similar to those seen in oocytes and embryos from ageing mice. Therefore, one of the possible mechanisms for the low fecundity of aged animals is presumably attributable to increased ROS levels resulting from mitochondrial dysfunction (Tarin, 1995; Wilding *et al.*, 2001; Thouas *et al.*, 2005) and to decreased antioxidant mechanisms (Friedman *et al.*, 1997; Van Blerkom *et al.*, 1997; Carbone *et al.*, 2003). It may be easier for the ROS level to reach a harmful level in ageing oocytes and embryos than in young oocytes and embryos, leading to a lower potential for embryo development and more frequent developmental arrest *in vitro*. This suggests that the culture environment for oocytes and embryos from aged animals needs more attention (Thouas *et al.*, 2005). Accordingly, attempts

have been made to reduce the level of ROS in oocytes, embryos and their immediate environment in order to improve embryo development. Tarin *et al.* (1998, 2002) have shown that oral administration of antioxidant neutralizes the disturbing effects of ageing on the segregation of chromosomes during the first meiotic division and the distribution of chromosomes in the metaphase II spindle. However, whether *in vitro* supplementation with antioxidants counteracts the negative effects of female ageing has not been well investigated. To our knowledge, this is the first report that has demonstrated that embryos from ageing mice show reduced growth *in vitro*, and that the presence of erythrocytes could improve embryo development.

As shown in Table II, addition of erythrocytes to the culture media of IVF embryos did not accelerate the development of embryos from young mice, suggesting that the addition of erythrocytes is only useful for embryos from ageing mice. However, the reason as to why the addition of erythrocytes did not have a significant effect on embryo development in the young group remains unclear. We conjecture that the scavenging constituents that originally existed in the sequential medium that we used eliminated the harmful ROS generated *in vitro*. Similar results, with no significant difference, have been reported for other studies in which SOD or its equivalent was added and a medium containing other scavenging constituents, such as EDTA, was used (Orsi and Leese, 2001; Ali *et al.*, 2003). We consider erythrocytes to be effective in the ageing mice because the amount of ROS generated overwhelmed the amount that the scavenging constituents alone were able to eliminate.

Although quite a few studies have reported that the rates of development to the blastocyst stage are low in embryos from ageing females compared with those from young females, some of these studies have also reported no significant difference in implantation rate between ageing and young groups when the blastocyst is obtained *in vitro* (Pantos *et al.*, 1999; Shapiro *et al.*, 2002). However, precise inspection to check for chromosomal aberration in addition to implantation and pregnancy rates is required for blastocysts obtained by adding erythrocytes to ageing embryos. Further studies are required to find out whether this method works similarly in other species, including humans, and the mechanism should also be investigated. When the mechanism is clarified, the addition of erythrocytes can be used to rescue embryos with low potential from ageing individuals, in which development may arrest in the normal *in vitro* environment. Increasing numbers of women are delaying childbearing until the end of the third and sometimes the fourth decade of life, and therefore there is a strong need for the clinical application of this technique.

Acknowledgements

We are grateful to Mr M. Kaneko at Asahi Medical Co., Ltd. for the generous donation of leukocyte depletion filters. We wish to thank Mr. F. Kojima for technical assistance and PhD. S. Watanabe for the advice and expertise.

References

Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003;**79**:829–843.

Ali AA, Bilodeau JF, Sirard MA. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology* 2003;**59**:939–949.

Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, Sunde A, Templeton A, Van Steirteghem A, Cohen J *et al.* Fertility and ageing. *Hum Reprod Update* 2005;**11**:261–276.

Battaglia DE, Goodwin P, Klein NA, Soules MR. Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. *Hum Reprod* 1996;**11**:2217–2222.

Bavister BD. Culture of preimplantation embryos: facts and artifacts. *Hum Reprod Update* 1995;**1**:91–148.

Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R, Amicarelli F. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. *Mol Hum Reprod* 2003;**9**: 639–643.

Dinara S, Sengokku K, Tamate K, Horikawa M, Ishikawa M. Effects of supplementation with free radical scavengers on the survival and fertilization rates of mouse cryopreserved oocytes. *Hum Reprod* 2001;**16**:1976–1981.

Friedman CI, Danforth DR, Herbosa-Encarnacion C, Arbogast L, Alak BM, Seifer DB. Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. *Fertil Steril* 1997;**68**:607–612.

Fujitani Y, Kasai K, Ohtani S, Nishimura K, Yamada M, Utsumi K. Effect of oxygen concentration and free radicals on in vitro development of in vitro-produced bovine embryos. *J Anim Sci* 1997;**75**:483–489.

Goto Y, Noda Y, Mori T, Nakano M. Increased generation of reactive oxygen species in embryos cultured in vitro. *Free Radic Biol Med* 1993;**15**:69–75.

Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;**7**:175–189.

Harman D. Ageing: theory based on free radical and radiation chemistry. *J Gerontol* 1956;**11**:298–300.

Harman D. The aging process. *Proc Natl Acad Sci USA* 1981;**78**:7124–7128.

Janny L, Menezo YJ. Maternal age effect on early human embryonic development and blastocyst formation. *Mol Reprod Dev* 1996;**45**:31–37.

Keefe DL. Ageing and infertility in women. *Med Health R I* 1997;**80**:403–405.

Keefe DL, Niven-Fairchild T, Powell S, Buradagunta S. Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil Steril* 1995;**64**:577–583.

Kim JH, Lee SH, Kim S, Jeong YW, Koo OJ, Hashem MD, Park SM, Lee EG, Hossein MS, Kang SK *et al.* Embryotrophic effects of ethylenediaminetetraacetic acid and hemoglobin on in vitro porcine embryos development. *Theriogenology* 2006;**66**:449–455.

Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology* 2004;**62**:1186–1197.

Koehler KE, Hawley RS, Sherman S, Hassold T. Recombination and nondisjunction in humans and flies. *Hum Mol Genet* 1996;**5**:1495–1504.

Legge M, Sellens MH. Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. *Hum Reprod* 1991;**6**:867–871.

Liu L, Keefe DL. Cytoplasm mediates both development and oxidation-induced apoptotic cell death in mouse zygotes. *Biol Reprod* 2000;**62**:1828–1834.

Liu L, Trimarchi JR, Keefe DL. Thiol oxidation-induced embryonic cell death in mice is prevented by the antioxidant dithiothreitol. *Biol Reprod* 1999; **61**:1162–1169.

Liu L, Trimarchi JR, Keefe DL. Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes. *Biol Reprod* 2000;**62**: 1745–1753.

Matsuoka I, Fujino Y, Ogita S, Inoue M. Impact of erythrocytes on mouse embryonal development in vitro. *FEBS Lett* 1995;**371**:297–299.

Motoyama S, Saito S, Inaba H, Kitamura M, Minamiya Y, Suzuki H, Saito R, Kamata S, Nakae H, Ogawa J. Red blood cells attenuate sinusoidal endothelial cell injury by scavenging xanthine oxidase-dependent hydrogen peroxide in hyperoxic perfused rat liver. *Liver* 2000;**20**:200–208.

Musoh A, Yamamasu S, Tokuyama O, Nakamura Y, Musoh H, Fujino Y, Ishiko O, Ogita S. Promotion of human blastocyst formation by red cell hemolysate. *Osaka City Med J* 2002;**48**:39–44.

Navot D, Bergh PA, Williams MA, Garrisi GJ, Guzman I, Sandler B, Grunfeld L. Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. *Lancet* 1991;**337**:1375–1377.

Nohl H, Jordan W, Hegner D. Identification of free hydroxyl radicals in respiring rat heart mitochondria by spin trapping with the nitrone DMPO. *FEBS Lett* 1981;**123**:241–244.

Olson SE, Seidel GE, Jr. Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. *Biol Reprod* 2000;**62**:248–252.

Orsi NM, Leese HJ. Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. *Mol Reprod Dev* 2001;**59**: 44–53.

Pantos K, Athanasiou V, Stefanidis K, Stavrou D, Vaxevanoglou T, Chronopoulou M. Influence of advanced age on the blastocyst development rate and pregnancy rate in assisted reproductive technology. *Fertil Steril* 1999;**71**:1144–1146.

Park SE, Chung HM, Ko JJ, Lee BC, Cha KY, Lim JM. Embryotropic role of hemoglobin and ethylenediaminetetraacetic acid in preimplantation development of ICR mouse 1-cell embryos. *Fertil Steril* 2000;**74**:996–1000.

Park SE, Chung HM, Cha KY, Hwang WS, Lee ES, Lim JM. Establishment of an animal embryo culture system containing various embryotropins and its efficacy for culturing ICR mouse one-cell embryos derived in vivo or in vitro. *Fertil Steril* 2001;**76**:167–174.

Richards RS, Roberts TK, Dunstan RH, McGregor NR, Butt HL. Erythrocyte antioxidant systems protect cultured endothelial cells against oxidant damage. *Biochem Mol Biol Int* 1998;**46**:857–865.

Richards RS, Roberts TK, McGregor NR, Dunstan RH, Butt HL. The role of erythrocytes in the inactivation of free radicals. *Med Hypotheses* 1998;**50**:363–367.

Salmen JJ, Skufca F, Matt A, Gushansky G, Mason A, Gardiner CS. Role of glutathione in reproductive tract secretions on mouse preimplantation embryo development. *Biol Reprod* 2005;**73**:308–314.

Shapiro BS, Richter KS, Harris DC, Daneshmand ST. Influence of patient age on the growth and transfer of blastocyst-stage embryos. *Fertil Steril* 2002;**77**:700–705.

Tarin JJ. Aetiology of age-associated aneuploidy: a mechanism based on the 'free radical theory of ageing'. *Hum Reprod* 1995;**10**:1563–1565.

Tarin JJ. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol Hum Reprod* 1996;**2**:717–724.

Tarin JJ, Vendrell FJ, Ten J, Cano A. Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. *Mol Hum Reprod* 1998;**4**: 281–288.

Tarin JJ, Perez-Albala S, Cano A. Oral antioxidants counteract the negative effects of female aging on oocyte quantity and quality in the mouse. *Mol Reprod Dev* 2002;**61**:385–397.

Thouas GA, Trounson AO, Jones GM. Effect of female age on mouse oocyte developmental competence following mitochondrial injury. *Biol Reprod* 2005;**73**:366–373.

Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod* 1995;**10**:415–424.

Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Hum Reprod* 1997;**12**:1047–1055.

Wang X, Falcone T, Attaran M, Goldberg JM, Agarwal A, Sharma RK. Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertil Steril* 2002;**78**:1272–1277.

Wei YH, Lu CY, Lee HC, Pang CY, Ma YS. Oxidative damage and mutation to mitochondrial DNA and age-dependent decline of mitochondrial respiratory function. *Ann N Y Acad Sci* 1998;**854**:155–170.

Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod* 2001;**16**:909–917.

Submitted on December 2, 2006; resubmitted on April 15, 2008; accepted on April 21, 2008

ELSEVIER

Journal of Reproductive Immunology xxx (2009) xxx–xxx

JOURNAL OF REPRODUCTIVE IMMUNOLOGY

www.elsevier.com/locate/jreprimm

Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes

Hideto Yamada [a,*], Tatsuya Atsumi [b], Gen Kobashi [c], Chikako Ota [d], Emi H. Kato [a], Noriko Tsuruga [a], Kaori Ohta [c], Shinsuke Yasuda [b], Takao Koike [b], Hisanori Minakami [a]

[a] *Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Kita-ku N15 W7, Sapporo 060-8638, Japan*
[b] *Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan*
[c] *Molecular Biostatistics Research Team, Research Center for Charged Particle Therapy, National Institute of Radiological Science, Chiba, Japan*
[d] *Department of Obstetrics and Gynecology, Hirosaki University School of Medicine, Hirosaki, Japan*

Received 4 June 2008; received in revised form 5 November 2008; accepted 11 November 2008

Abstract

Antiphospholipid antibody (aPL) is associated with thromboembolism. There is scant evidence of a relationship between the aPL profile and serious adverse pregnancy outcome. The aim of this study was to assess whether aPL measurements during early pregnancy were useful in predicting a serious adverse pregnancy outcome. In this prospective study, we measured aPLs, including lupus anticoagulant (LA), IgG, IgM, IgA anticardiolipin antibody (aCL), IgG, IgM phosphatidylserine-dependent antiprothrombin antibody, and IgG kininogen-dependent antiphosphatidylethanolamine antibody (aPE) during the first trimester in a consecutive series of 1155 women. The 99th percentile cut-off values in each aPL were determined using samples from 105 women who did not exhibit any pregnancy morbidity. We assessed the predictive risk of a serious adverse pregnancy outcome adjusted for confounding factors. We found that IgG aCL was asssociated with developing pregnancy-induced hypertension (PIH) (odds ratio 11.4, 95% CI 2.7–48); IgG aPE with PIH (8.3, 2.4–29), severe PIH (20.4, 4.5–91), and premature delivery (PD) (12.7, 3.1–50); and LA with PD (11.0, 2.8–44) and low birth weight (8.0, 2.1–31). The combinations of IgG aPE plus IgG aCL (17.5, 4.7–66.7) or IgG aPE plus LA (22.2, 5.4–909) measurements predicted severe PIH with 30.8% sensitivity and 99.2% specificity. We conclude that aPL measurements during early pregnancy may be useful in predicting adverse pregnancy outcome.

Keywords: Antiphospholipid antibody; Pregnancy-induced hypertension; Premature delivery; Fetal loss; Fetal growth restriction

1. Introduction

Pregnancy-induced hypertension (PIH) is a major cause of mortality and morbidity during pregnancy and childbirth and is a multifactorial disease with genetic and environmental factors involved in its etiology. Severe PIH and pre-eclampsia can lead to multiple organ failure including the cardiovascular system, central nervous system, coagulation, liver, and kidneys. The association between antiphospholipid antibodies (aPLs) and the risk of PIH, pre-eclampsia, fetal growth restriction (FGR), or premature delivery (PD) still remains

* Corresponding author. Tel.: +81 11 706 7720; fax: +81 11 706 7711.
E-mail address: yhideto@med.hokudai.ac.jp (H. Yamada).

0165-0378/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jri.2008.11.001

Please cite this article in press as: Yamada, H., et al., Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. (2009), doi:10.1016/j.jri.2008.11.001

controversial. In retrospective case-control studies, it was found that women with a history of severe pre-eclampsia or hemolysis-elevated liver enzymes-low platelets (HELLP) syndrome frequently tested positive for lupus anticoagulant (LA) and anticardiolipin antibody (aCL) (Van Pampus et al., 1999; Von Tempelhoff et al., 2000).

However, prospective studies assessing associations between aPLs and PIH, pre-eclampsia or other pregnancy adverse outcomes found conflicting results. Studies conducted in the 1990s noted that pre-eclampsia was associated with the presence of LA (Pattison et al., 1993), aCL (Pattison et al., 1993; Yasuda et al., 1995), β_2-glycoprotein-dependent anticardiolipin antibody (aCLβ_2GPI) (Katano et al., 1996), and anti-β_2 glycoprotein-I antibody (aβ_2GPI; Faden et al., 1997). Similarly, fetal loss and FGR were associated with the presence of aCL (Yasuda et al., 1995; Katano et al., 1996). Recent prospective studies, however, failed to show an association between pre-eclampsia and the presence of LA (Dreyfus et al., 2001), aCL (Branch et al., 2001; Dreyfus et al., 2001; Lee et al., 2003b) or aβ_2GPI (Lee et al., 2003b). PIH (Lynch et al., 1999) and HELLP syndrome (Lee et al., 2003b) were not associated with the presence of aCL or aβ_2GPI.

Currently, a wide variety of aPLs in the human blood can be measured by laboratory diagnostic assays, each of which requires evaluation with regard to whether there is an association with obstetric events. The aim of this prospective study was to assess whether aPL measurements during early pregnancy were useful in predicting serious adverse pregnancy outcome in the Japanese population, which has a relatively homogeneous genetic background.

2. Materials and methods

2.1. Subjects

This prospective study, designated Sapporo Multiple Antiphospholipid Testing for the Prediction of Obstetric Outcome study (SAPPORO study) was performed in the city of Sapporo, Japan, and conducted with informed consent from all of the subjects, and was approved by the institutional ethics board of Hokkaido University Graduate School of Medicine. The peripheral blood was obtained at 8–14 weeks' gestation (GW) from 1220 consecutive Japanese women with living fetuses who visited the Hokkaido University Hospital or an affiliate hospital.

Measurements were made of aPLs including lupus anticoagulant (LA), IgG, IgM, IgA anticardiolipin antibody (aCL), IgG, IgM phosphatidylserine-dependent antiprothrombin antibody (aPS/PT), and IgG kininogen-dependent antiphosphatidylethanolamine antibody (aPE). The appropriate cut-off values of each aPL during the first trimester were determined using data obtained from the first 105 women who subsequently delivered a healthy neonate without pregnancy-related morbidity. In this study protocol, when women had a history of recurrent pregnancy loss (RPL) or thromboembolism with a positive test for LA or aCL, they underwent low-dose aspirin therapy (81 mg/day) until delivery. The ethics board directed this intervention in patients who were compatible with the original antiphospholipid syndrome (APS) criteria (Wilson et al., 1999); otherwise the study would not have been approved.

Women who were subsequently found to have a fetal anomaly or multiple pregnancy, and those whose pregnancies were terminated by an induced abortion were excluded from the analysis. We assessed risks of PIH, severe PIH, pre-eclampsia, fetal growth restriction (FGR, <10th percentile and <−1.5 S.D. based on gestational age, parity and fetal sex), premature delivery (PD, <34 and <37 GW), low birth weight (LBW, <2500 g), and fetal loss (fetal death after the blood sampling) with lifestyle-related confounding factors including maternal age, parity, preconception body mass index (BMI), cigarette smoking (preconception/during pregnancy), and drinking alcohol (preconception/during pregnancy). The mean age at blood sampling was 30.6 (S.D. 4.7) ranging from 16 to 44 years of age, and the mean preconception BMI was 20.9 (3.3) ranging from 14.9 to 44.5 kg/m^2.

Pregnancy-induced hypertension was diagnosed during pregnancy when hypertension (systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg) was detected after 20 GW. Severe PIH was diagnosed when at least one of the following criteria was met:

(1) Blood pressure ≥160/110 mmHg after 20 GW, regardless of the complication of proteinuria defined as urinary excretion of 300 mg protein/day,
(2) Blood pressure ≥140/90 mmHg after 20 GW complicated by proteinuria ≥2.0 g/day.

Pre-eclampsia was diagnosed when hypertension (≥140/90 mmHg) and proteinuria (≥300 mg/day) were detected after 20 GW.

Four women who had a history of RPL or thromboembolism with a positive test for LA or aCL underwent low-dose aspirin therapy. Of the four women, two received additional heparin therapy with informed consent.

Please cite this article in press as: Yamada, H., et al., Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. (2009), doi:10.1016/j.jri.2008.11.001

2.2. Antiphospholipid antibody measurement

Lupus anticoagulant was determined by clotting assays using an opto-mechanical coagulation analyzer (ST art 4, Diagnostica Stago, Asnières, France) according to the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibody guidelines. Kaolin clotting time (KCT) and activated Partial Thromboplastin Time (aPTT) using a sensitive reagent with a low phospholipid concentration (PTT-LA, Diagnostica Stago) were used for a screening and mixing procedure, followed by confirmation with the Staclot LA kit (Diagnostica Stago) or Dilute Russell's Viper Venom Time (dRVVT), using the LA test Gradipore OR (Gradipore, Frenchs Forest, NSW, Australia). LA was considered to be present when screening clotting time was prolonged and at least one of these tests was positive.

IgG, IgM, IgA aCL was measured according to the standard aCL ELISA (Harris et al., 1987). IgG and IgM aPS/PT were detected by ELISA, as previously described (Atsumi et al., 2000). Briefly, non-irradiated microtiter plates (Sumilon type S, Sumitomo Bakelite, Tokyo, Japan) were coated with 30 ml of 50 mg/ml phosphatidylserine (Sigma Chemical Co., St. Louis, USA), and dried overnight at 4 °C. To avoid nonspecific binding of proteins, wells were blocked with 150 ml of Tris-buffered saline (TBS) containing 1% fatty-acid free bovine serum albumin (BSA, A-6003, Sigma) and 5 mM $CaCl_2$ (BSA-Ca). After three washes in TBS containing 0.05% Tween 20 (Sigma) and 5 mM $CaCl_2$ (TBS-Tween-Ca), 50 ml of 10 ml/ml human prothrombin (Diagnostica Stago, Asnieres, France) in BSA-Ca was added to half of the wells in the plates and the same volume of BSA-Ca alone (as a blank sample) to the other half. After a 1-h incubation period at 37 °C, the plates were washed and 50 ml of serum diluted in BSA-Ca in 1:100 was added in duplicate. Plates were incubated for 1 h at room temperature, followed by alkaline phosphatase (ALP)-conjugated goat anti-human IgG and substrate. The optical density (OD) of the wells coated solely with phosphatidylserine was subtracted from that of wells containing phosphatidylserine-prothrombin. The titer of each sample was derived from the standard curve according to dilutions of the positive control.

IgG kininogen-dependent antiphosphatidylethanolamine antibody (aPE) was tested by ELISA, established by Sugi et al., using adult bovine plasma that contained a high concentration of intact kininogen as a sample diluent (Sugi et al., 1999). When the results of a positive test measured with phosphatidylethanolamine and a negative test without phosphatidylethanolamine were obtained, the presence of aPE was determined (Sugi et al., 1999).

2.3. Statistical analysis

Statistical analyses were performed to compare the number of serious adverse pregnancy outcomes in group of patients positive for aPLs or confounding factors with that in the negative group. Differences were analyzed statistically by the Chi-squared test (d.f. = 1). Fisher's exact test was used when an observed number was ≤ 5. Relative risks (RR) and 95% confidence intervals (95% CI) were calculated via univariate analyses. The aPLs that were significantly ($P < 0.05$) associated with serious adverse pregnancy outcomes in the univariate analysis were further assessed by a multivariate analysis.

A multiple logistic regression model was used to evaluate the associations between aPLs and serious adverse pregnancy outcomes with adjustment for maternal age, parity, BMI, smoking, and drinking alcohol; adjusted odds ratios and 95% CI were calculated. All statistical analyses were conducted using a statistical analysis system package (SAS ver. 9.1, SAS Institute Japan Ltd., Tokyo, Japan).

3. Results

A total of 65 cases with a fetal anomaly, multiple pregnancy, or induced abortion after the blood sampling were excluded from the analysis. In 1155 women, the frequencies of the lifestyle-related confounding factors were as follows: ≥35 years old, 19.2%; multiparity, 44.6%; BMI ≥25 kg/m^2, 8.6%; smoking before pregnancy, 27.3%; smoking during pregnancy, 12.2%; drinking alcohol before pregnancy, 20.6%; and drinking alcohol during pregnancy, 3.2%.

Fourteen (1.2%) of the 1155 pregnancies suffered a fetal loss including spontaneous abortion ($n = 12$; seven at 8–14 GW, five at 15–19 GW) at less than 22 GW and intrauterine fetal death ($n = 2$; at 31–34 GW) at 23 or more GW. The other 1141 pregnancies ended in live births of infants with a mean weight of 2,969 (S.D. 498) g (490–4558 g) at a mean of 38.8 (S.D. 2.6) GW (27–42 GW). Of the 1155 pregnancies, the following serious adverse pregnancy outcomes occurred, corresponding to the normal frequencies in the Japanese population: PIH ($n = 36$; 3.1%), severe PIH ($n = 13$; 1.1%), pre-eclampsia ($n = 18$; 1.6%), FGR at <10th percentile ($n = 112$; 9.7%), FGR <−1.5 S.D. ($n = 64$; 5.5%), PD at <37 GW ($n = 94$; 8.1%), PD at <34 GW ($n = 17$; 1.5%), and LBW ($n = 134$; 11.6%).

Four women who had a history of RPL or thromboembolism with a positive test for LA or aCL underwent antithrombotic therapy. Of the four, one woman developed severe PIH. Two pregnancies ended in PD. The

Please cite this article in press as: Yamada, H., et al., Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. (2009), doi:10.1016/j.jri.2008.11.001

Table 1
Confounding factors as risk factors for serious adverse pregnancy outcomes determined by univariate analysis.

Serious adverse pregnancy outcome	Confounding factor	Prevalence of complications in women with the factor	*P*-value	Relative risk	95% CI
PIH	Multiparity	1.9%	0.035	0.47	0.23–0.99
	Body mass index $\geq 25\ kg/m^2$	12.1%	<0.0001	5.3	2.6–11.0
Severe PIH	Body mass index $\geq 25\ kg/m^2$	4.0%	0.020	4.7	1.4–15.6
Pre-eclampsia	Body mass index $\geq 25\ kg/m^2$	5.1%	0.015	4.1	1.4–11.7
Fetal growth restriction (<10th percentile)	Multiparity	14.7%	<0.0001	2.1	1.4–3.2
	Smoking during Pregnancy	16.9%	0.013	1.7	1.1–2.9
Fetal growth restriction (<−1.5 S.D.)	Smoking during Pregnancy	13.1%	0.0004	2.6	1.4–4.6
Premature delivery (<37 weeks)	Drinking before pregnancy	14.3%	0.0008	2.0	1.3–3.1
Fetal loss	Smoking during pregnancy	2.8%	0.019	5.5	1.5–20.7
	Drinking before pregnancy	2.1%	0.027	4.5	1.2–16.8

PIH, pregnancy-induced hypertension.

other two pregnancies ended in normal deliveries without adverse pregnancy outcome.

The 99th percentile cut-off values of aPLs were determined by data obtained from the first 105 pregnant women who had no adverse pregnancy outcomes. These 99th percentile cut-off values were: LA screening, represented by KCT in a 1:4 mixed sample, the ratio (1.33) of clotting time over that of normal plasma (the presence of LA was subsequently confirmed if the clotting time of mixed plasma was more than that level); IgG aCL 9.6 GPL, IgM aCL 9.9 MPL, and IgA aCL 24 APL; IgG aPS/PT 3.4 U/ml and IgM aPS/PT 92 U/ml; and IgG aPE 0.4918-OD_{405}. The positive frequencies of aPLs with these 99th percentile cut-off values in the 1155 women were as follows: LA, 0.8%; IgG aCL 1.1%, IgM aCL 2.3%, IgA aCL 0.5%; IgG aPS/PT 0.2%, IgM aPS/PT 0.2%; IgG aPE, 2.1%. The frequency of a positive test for at least one aPL, designated as "any aPL," was found to be 6.4%. The frequency of a positive test for two or more aPLs among LA, aCL, aPS/PT, and aPE, designated as "multi-positive," was 0.3%; and the frequency of double-positive tests for LA and IgG, IgM, or IgA aCL, designated as "double-positive," was 0.3%

We found that confounding factors, i.e., lifestyle-related factors, were significantly associated with serious adverse pregnancy outcomes (Table 1). A high BMI was associated with PIH, severe PIH, and pre-eclampsia; smoking was associated with FGR and fetal loss; drinking alcohol was associated with PD and fetal loss; and parity was associated with PIH and FGR.

Table 2 presents the risk factors for PIH and severe PIH determined by a univariate analysis. IgG aCL, IgG

Table 2
Antiphospholipid antibodies as risk factors for (severe) pregnancy-induced hypertension determined by univariate analysis..

aPL	Prevalence of PIH in positive population	*P*-value	Relative risk (95% CI)	Prevalence of severe PIH in positive population	*P*-value	Relative risk (95% CI)
LA	11.1%			11.1%	0.097	10.6 (1.2–90.3)
IgG aCL	23.1%	0.006	8.0 (2.2–29.4)	7.7%		
IgM aCL	3.7%			0		
IgA aCL	0			0		
IgG aPS/PT	0			0		
IgM aPS/PT	0			0		
IgG aPE	16.7%	0.005	5.9 (1.9–18.0)	12.5%	0.002	14.1 (3.7–54.6)
Any aPL	10.8%	<0.0001	4.2 (1.8–9.5)	5.4%	0.007	6.5 (1.9–21.5)
Multi-positive	25.0%	0.119	8.2 (0.90–75.5)	25.0%	0.040	23.9 (2.5–230.3)
Double-positive (LA and aCL)	33.3%	0.091	11.0 (1.1–108.1)	33.3%	0.003	31.9 (3.1–329.5)

aPL, antiphospholipid antibody; LA, lupus anticoagulant; aCL, anticardiolipin antibody; aPS/PT, phosphatidylserine-dependent antiprothrombin antibody; aPE, kininogen-dependent antiphosphatidylethanolamine antibody; PIH, pregnancy-induced hypertension.

Please cite this article in press as: Yamada, H., et al., Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. (2009), doi:10.1016/j.jri.2008.11.001

Table 3
Antiphospholipid antibodies as risk factors for premature delivery (<37 and <34 weeks' gestation) determined by univariate analysis).

aPL	Prevalence of premature delivery (<37 weeks) in positive population	*P*-value	Relative risk (95% CI)	Prevalence of premature delivery (<34 weeks) in positive population	*P*-value	Relative risk (95% CI)
LA	55.6%	<0.0001	6.6 (2.2–20.1)	0		
IgG aCL	23.1%			7.7%		
IgM aCL	7.7%			0		
IgA aCL	16.7%			0		
IgG aPS/PT	50.0%			0		
IgM aPS/PT	0			0		
IgG aPE	18.2%			12.5%	0.004	10.1 (2.7–37.5)
Any aPL	17.1%	0.011	2.1 (1.1–4.0)	5.4%	0.020	4.5 (1.4–14.1)
Multi-positive	50.0%	0.041	5.8 (1.04–31.9)	0		
Double-positive (LA and aCL)	66.7%	0.022	7.7 (1.3–46.6)	0		

aPL, antiphospholipid antibody; LA, lupus anticoagulant; aCL, anticardiolipin antibody; aPS/PT, phosphatidylserine-dependent antiprothrombin antibody; aPE, kininogen-dependent antiphosphatidylethanolamine antibody; PIH, pregnancy-induced hypertension.

aPE, and any aPL increased the risk of PIH, while IgG aPE, any aPL, multi-positive, and double-positive, increased the risk of severe PIH (Table 2). We found weak associations between pre-eclampsia and aPE IgG (RR 5.9, 95% CI 1.3–27.1), but with borderline significance ($p = 0.052$).

Table 3 presents risk factors for PD at <37 GW and PD at <34 GW. LA, any aPL, multi-positive, and double-positive, increased a risk of PD at <37 GW, while IgG aPE and any aPL increased the risk of PD at <34 GW. LA (RR 4.6, 95% CI 1.5–13.9, $p = 0.002$) increased a risk of LBW. None of the aPLs was associated with a risk of FGR <10th percentile, FGR <−1.5 S.D., or fetal loss.

A multiple logistic regression model with adjustment for confounding factors demonstrated that aPLs were risk factors for serious adverse pregnancy outcomes including PIH, severe PIH, PD, and LBW (Table 4).

To determine whether a positive test for any of the two aPLs effectively predicted the most serious adverse pregnancy outcome (i.e., PIH and severe PIH), we searched for a combination of two types of aPLs. According to Benjamini and Hochberg's correction to control the false discovery rate in a multiple comparison, the only combination of IgG aPE plus IgG aCL measurement was found to be a significant combination that predicted PIH with 19.4% sensitivity and 97.4% specificity (OR 10.6,

Table 4
Antiphospholipid antibodies as risk factors for serious adverse pregnancy outcomes determined by multivariate analysis.

Adverse pregnancy outcome	aPL	Odds ratio	95% CI
PIH	IgG aCL	11.4	2.7–47.6
	IgG aPE	8.3	2.4–28.6
	Any aPL	5.5	2.3–13.5
Severe PIH	IgG aPE	20.4	4.5–90.9
	Any aPL	8.1	2.2–29.4
	Multi-positive	143	9.8–1000
	Double-positive (LA and aCL)	250	11.1–1000
Premature delivery (<37 weeks)	LA	11.0	2.8–43.5
	Any aPL	2.3	1.1–4.4
	Multi-positive	11.6	1.5–90.9
	Double-positive (LA and aCL)	22.2	1.9–250
Premature delivery (<34 weeks)	IgG aPE	12.7	3.1–50.0
	Any aPL	4.5	1.4–14.9
Low birth weight	LA	8.0	2.1–31.3
	Double-positive (LA and aCL)	13.7	1.2–167

aPL, antiphospholipid antibody; PIH, pregnancy-induced hypertension; aCL, anticardiolipin antibody; aPE, kininogen-dependent antiphosphatidylethanolamine antibody; LA, lupus anticoagulant.

Please cite this article in press as: Yamada, H., et al., Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. (2009), doi:10.1016/j.jri.2008.11.001

95% CI 4.0–28.6, $p < 0.0001$). Similarly, the combinations of IgG aPE plus IgG aCL, and IgG aPE plus LA predicted severe PIH with 30.8% sensitivity and 99.2% specificity (OR 17.5, 95% CI 4.7–66.7, $p < 0.0001$), and with 30.8% sensitivity and 99.2% specificity (OR 22.2, 95% CI 5.4–909, $p < 0.0001$). Any combination of three or more kinds of aPL measurement did not significantly improve the sensitivity and specificity for the prediction of PIH or severe PIH. When one of the lifestyle-related factors, high BMI ($\geq 25\ kg/m^2$), was added to the aPL combination of IgG aPE plus IgG aCL, the presence of high BMI or a positive test for the aPLs predicted severe PIH with higher sensitivity (53.8%) and specificity (99.4%; OR 11.2, 95% CI 3.4–37.0, $p < 0.0001$).

4. Discussion

In this study, we found that lifestyle-related confounding factors were significantly associated with the risks of a serious adverse pregnancy outcome, including high BMI with PIH, severe PIH, and pre-eclampsia; cigarette smoking with FGR and fetal loss; drinking alcohol with PD and fetal loss; and primiparity with PIH and multiparity with FGR. It is well documented that obesity, primiparity, and age ≥35 years are risk factors for PIH (Poole, 1997) and pre-eclampsia (ACOGCOP, 2002; Lain and Roberts, 2002). It has also been reported that smoking increases the risks of FGR and fetal loss (Ness et al., 1999; Lindbohm et al., 2002) and drinking alcohol increases the risk of FGR, PD (Windham et al., 1995), and fetal loss (Kesmodel et al., 2002). Our findings are comparable to the aforementioned, suggesting that our study participants had little or no deviation in lifestyle from that of the standard population of pregnant women. In this study, multiparity was related to FGR <10th percentile, but not to FGR <−1.5 S.D. We speculate that in our participants, some lifestyle factors, such as diet for multiparas who were city dwellers, might affect fetal growth. By adjusting for the confounding factors described above, we determined that positive tests for aPL measurements in early pregnancy were risk factors for the occurrence of PIH, severe PIH, PD, and LBW later in the pregnancy.

We found that IgG aCL, IgG aPE, and multi-positive aPL was a risk factor for PIH or severe PIH; and that double-positive aPL (LA and aCL) was a risk factor for severe PIH. To the best of our knowledge, this is the first evidence regarding the association between the multi-/double-positive aPL and PIH. Similarly, recent studies have suggested that the multi-positive test is associated with a more severe course of APS disease, increasing significantly the rate of thrombosis (Detkova et al., 1999; Lee et al., 2003a; Obermoser et al., 2003, 2004). Thus, it is likely that multi-/double-positive aPL predicts a higher risk of PIH and severe PIH as well. Pregnant women with multiple/double aPL should be more carefully managed during pregnancy.

In the present study, LA and double-positive aPL were associated with PD at <37 GW and LBW. In this study design, there was the limitation that the physicians knew the results of LA and aCL measurements in women who had a history of thromboembolism or RPL because these aPLs are included in the laboratory findings of the APS criteria (Wilson et al., 1999; Miyakis et al., 2006). The knowledge of the presence of these aPLs may have influenced the physician in favor of an early pregnancy termination. The possibility of this bias cannot be excluded.

Little is known about the relationship between thromboembolism/adverse pregnancy outcome and aPE; however, recent studies have reported that there are associations. More specifically, aPE was frequently detected in patients with unexplained recurrent early fetal loss, mid-to-late fetal loss, unexplained thrombosis, systemic lupus erythematosus, heart valvulopathies, and livedo reticularis (Gris et al., 2000; Sanmarco et al., 2001; Balada et al., 2001; Yamada et al., 2003; Sugi et al., 2004). In the current study, it was demonstrated by a multivariate analysis that aPE increased the risk of PIH, severe PIH, and PD at <34 GW. All women who developed severe PIH with aPE ended up undergoing induced PD at <34 GW by cesarean section (data not shown). We measured the kininogen-dependent antiphosphatidylethanolamine antibody that probably binds to kininogen as a cofactor (Sugi et al., 1999). The kallikrein–kinin system is involved in blood pressure control and angiogenesis. Tissue kallikrein cleaves low-molecular-weight kininogen substrate to produce the vasodilator Lys-bradykinin, whereas plasma kallikrein forms bradykinin (BK) from high-molecular-weight kininogen (HK). Kininogen-deficient rats are susceptible to the development of salt-induced hypertension (Majima et al., 1994), and the in vivo angiogenesis is suppressed (Hayashi et al., 2002). The pro-angiogenic effect of BK and HK has been demonstrated in both in vitro and in vivo studies (Guo and Colman, 2005). Therefore, we assume that aPE pathophysiologically causes impairment of fetoplacental angiogenesis and vessel development, which subsequently may predispose women to PIH. Alternatively, disruption of the kininogen cascade in the kallikrein–kinin system may reduce vasodilator production and cause a hypertensive disorder. Recently, a multicenter study demonstrated that aPE, but not LA or aCL, was closely associated with thrombosis, having

Please cite this article in press as: Yamada, H., et al., Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. (2009), doi:10.1016/j.jri.2008.11.001