4. Boue A, Gallano P. A collaborative study of the segregation of inherited chromosome structural rearrangements in 1356 prenatal diagnoses. Prenat Diagn 1984;4:45–67.
5. Sugiura-Ogasawara M, Ozaki Y, Sato T, Suzumori N, Suzumori K. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocations. Fertil Steril 2004;81:367–73.
6. Carp H, Feldman B, Oelsner G, Schiff E. Parental karyotype and subsequent live births in recurrent miscarriage. Fertil Steril 2004;81:1296–301.
7. Stephenson MD, Sierra S. Reproductive outcomes in recurrent pregnancy loss associated with a parental carrier of a structural chromosome rearrangement. Hum Reprod 2006;21:1076–82.
8. Guttenbach M, Engel W, Schmid M. Analysis of structural and numerical chromosome abnormalities in sperm of normal men and carriers of constitutional chromosome aberrations. A review. Hum Genet 1997;100:1–21.
9. Mastenbroek S, Twisk M, van Echten-Arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, et al. In vitro fertilization with preimplantation genetic screening. N Engl J Med 2007;357:9–17.
10. Escudero T, Abdelhadi I, Sandalinas M, Munne S. Predictive value of sperm fluorescence in situ hybridization analysis on the outcome of preimplantation genetic diagnosis for translocations. Fertil Steril 2003;79(Suppl 3):1528–34.

Midline uterine defect size is correlated with miscarriage of euploid embryos in recurrent cases

Mayumi Sugiura-Ogasawara, M.D., Ph.D.,[a] *Yasuhiko Ozaki, M.D., Ph.D.,*[a] *Tamao Kitaori, M.D., Ph.D.,*[a] *Kyoko Kumagai, M.D., Ph.D.,*[a] *and Sadao Suzuki, M.D., M.Sc., Ph.D.*[b]

[a] Department of Obstetrics and Gynecology; and [b] Department of Public Health, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan

Objective: To compare subsequent pregnancy outcomes after two or more miscarriages in patients with and without congenital uterine anomalies.
Design: Case-control study.
Setting: Nagoya City University Hospital.
Patient(s): A total of 42 patients with a bicornuate or septate uterus and 1528 with normal uteri.
Main Outcome Measure(s): The cumulative success rate for birth, abnormal chromosome karyotype rate in aborted concepti, and the predictive values of the height of the defect/length of the remaining uterine cavity ratio (D/C ratio).
Result(s): Of the total of 1676 patients, 54 (3.2%) had congenital uterine anomalies; 25 (59.5%) of the 42 patients with a bicornuate or septate uterus had a successful first pregnancy after examination, while this was the case for 1096 (71.7%) of the 1528 with normal uteri. There was no difference in the cumulative live-birth rate (78.0% and 85.5%) within the follow-up period. However, the rates for an abnormal chromosome karyotype in aborted concepti in cases with and without uterine anomalies were 15.4% (two of 13) and 57.5% (134 of 233), respectively, with the latter being significantly higher. The D/C ratio in the miscarriage group was also significantly greater than that for the live-birth group.
Conclusion(s): Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage and are associated with further miscarriage with a normal embryonic karyotype. The D/C ratio was found to have a predictive value for further miscarriages in recurrent cases. (Fertil Steril® 2009;■:■–■. ©2009 by American Society for Reproductive Medicine.)

Key Words: Bicornuate uterus, congenital uterine anomaly, recurrent miscarriage, septate uterus

Established causes of recurrent miscarriages are antiphospholipid antibodies (aPL), uterine anomalies, and chromosomal abnormalities in the embryo (1–3). Abnormal chromosomes in either partner, particularly translocations, are also risk factors (4). Regarding uterine anomalies, Raga et al. reported that patients (6.3%, 54 of 868; $P<.05$) with a history of two or more miscarriages had a significantly elevated incidence of Mullerian anomalies compared with fertile (3.8%, 49 of 1289) and sterile (2.4%, 25 of 1024) cases (2). The frequency of congenital uterine anomalies has been reported to be between 1.8% and 37.6% in women with a history of recurrent miscarriage, the variation largely depending on the methods of selection and criteria for diagnosis (5–7).

Thus, affected patients are offered surgery in an attempt to restore the uterine anatomy (8–16). The conclusion is that operations can increase successful pregnancies, but to our knowledge there have been no prospective studies comparing pregnancy outcomes between cases with and without surgery in patients with a history of recurrent miscarriage. Lee et al. reported a preoperative pregnancy loss rate of 77.4%, a 18.2% miscarriage rate, and a 77.3% uncomplicated delivery rate after hysteroscopic septum resection (14). However, it is inappropriate to simply make comparisons before and after surgery because the miscarriage rate before examination might be 100% but the subsequent success rate is never 0. The subsequent live-birth rate is expected to be 72% in recurrent miscarriage patients without abnormal chromosomes in either partner (17) and decreases with the number of previous miscarriages (3).

Information concerning the prognosis in women with congenital uterine anomalies with a history of recurrent miscarriage is limited. The present study was therefore conducted to assess the subsequent live-birth rate, comparing pregnancy outcome between cases with and without bicornis or septum in individuals with a history of recurrent miscarriage.

PATIENTS AND METHODS

We conducted a case-control study. We studied 1676 patients with a history of two or more (2–12) consecutive miscarriages whose subsequent pregnancies were ascertained at least once in our medical records. Hysterosalpingography (HSG), chromosome analysis for both partners, determination of aPL,

Received June 18, 2008; revised and accepted December 18, 2008.
M.S.-O. has nothing to disclose. Y.O. has nothing to disclose. T.K. has nothing to disclose. K.K. has nothing to disclose. S.S. has nothing to disclose.
This study was supported by the Ministry of Health, Labour and Welfare.
Reprint requests: Mayumi Sugiura-Ogasawara, Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Mizuho-ku, Nagoya, 4678601, Japan (FAX: 81-52-842-2269; E-mail: og.mym@med.nagoya-cu.ac.jp).

FLA 5.0 DTD ■ SEC CODE: RPL ■ fns24298 ■ 7 February 2009 ■ 3:45 pm ■ ce 11

including lupus anticoagulant and β2-glycoprotein I dependent anticardiolipin antibodies (18), and blood tests for hyperthyroidism, diabetes mellitus, and hyperprolactinemia were performed for all patients before subsequent pregnancy. All patients were examined between 1986 and 2007 at Nagoya City University Hospital.

Laparoscopy/laparotomy and/or magnetic resonance imaging (MRI) were performed to ascertain the type of anomaly (investigating both the uterine cavity and the external uterine contours) in accordance with the American Fertility Society classification of Mullerian anomalies (19–21). Tompkin's index was used to distinguish between arcuate uterus and mild septate or bicornuate uterus (22). A Tompkin's index >25% was the criterion for septate or bicornuate uterus. Patients desiring surgical treatment before subsequent pregnancies underwent a Jones metroplasty, Strassman metroplasty, or hysteroscopic transcervical resection (TCR; 8–10).

All pregnancy outcomes of 1676 patients were examined. Patients with at least one kind of aPL were treated with low-dose aspirin and heparin combined therapy. Gestational age was calculated from basal body temperature charts. Ultrasound was performed once or twice a week from 4 to 8 weeks' gestation. Dilation and curettage was performed on all patients diagnosed with miscarriage, and the karyotypes of aborted conceptuses were determined with the use of a standard G-banding technique. The study was approved by the Research Ethics Committee at Nagoya City University Medical School.

In the present study, [1] the prevalence of clear congenital uterine malformations such as septate uterus, bicornuate uterus, unicornuate uterus, and didelphys was examined; [2] the first pregnancy outcome after systematic examination for recurrent miscarriage was determined for both septate and bicornuate uterus cases, comparing patients with or without anomalies; [3] all pregnancy outcomes after systematic examination were also assessed, and the final live-birth rate/patient was calculated; [4] abnormal karyotype rates for aborted concepti at the first miscarriage after the ascertainment of uterine abnormalities were also compared between patients with and without congenital uterine anomalies; [5] the height of the defect/length of the remaining uterine cavity (D/C) ratios were calculated in cases with bicornuate and septate uterine and compared between miscarriages and live birth at the subsequent first pregnancy. We also ascertained whether the D/C ratio has predictive value for further miscarriage in recurrent miscarriage cases.

The analysis was carried out using the SAS system (SAS Institute Inc., Cary, NC) with receiver operating curve (ROC) analysis and logistic regression. $P<.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

One thousand six hundred seventy-six patients became pregnant after systematic examination for recurrent miscarriages. Of this total, 54 (3.2%) had congenital uterine anomalies, 38 with partial bicornis unicolli, 10 with a septum, five with a unicornis, and one with a didelphys. None of them had hypoplasis/agenesis or diethylstilboestrol (DES) drug-related anomalies. Two patients with a septate uterus and a bicornuate uterus also had translocations in either partner. The 94 patients who had structural chromosome abnormalities, including 73 translocations, in either partner, were excluded from the analysis.

One thousand five hundred twenty-eight patients had neither congenital uterine anomalies nor an abnormal chromosome karyotype in either partner; 75 patients exhibited persistent aPL and were treated with low-dose aspirin and heparin combined therapy.

One of the two patients with bicornuate uteri underwent a Jones metroplasty, and the other underwent a Strassman metroplasty (8, 9). One patient with a septum also received a Jones metroplasty, and hysteroscopic TCR was performed for the other four patients with septate uteri.

We compared pregnancy outcomes between 42 patients with septate or bicornuate uteri not undergoing surgery and 1528 patients without uterine anomaly. We found no differences in baseline characteristics between the two groups (Table 1).

Pregnancy Outcome

Subsequent pregnancy outcomes are summarized in Table 2. Twenty-five of the 42 patients with a septate or bicornuate uterus (59.5%) treated without any kind of surgery had a successful outcome, while this was the case for 1096 (71.7%) of the 1528 without congenital uterine anomalies at the subsequent first pregnancy ($P=.084$). Four of five patients with a septate uterus and 21 of 37 patients with a bicornuate uterus gave birth to live babies. There was one case with a bicornuate uterus who suffered from uterine rupture in the first trimester because of the limited capacity.

One patient received surgery after further miscarriage. Thus, 32 (78.0%) of 41 patients and 1307 (85.5%) of 1528 patients with and without uterine anomalies could cumulatively have a live baby within the follow-up period (P = not significant). Live-birth rates of patients with congenital uterine anomalies tended to be lower both at the first pregnancy after ascertainment and cumulatively. Final live-birth rates/person are also shown in Table 2.

Furthermore, rates for an abnormal chromosome karyotype in aborted concepti in cases with and without uterine anomalies were 15.4% (two of 13) and 57.5% (134 of 233), respectively, at the first pregnancy after ascertainment of uterine anomalies, the difference being highly significant (Fisher's exact probability test, $P=.006$).

One of five patients with a unicornuate uterus succeeded in having a baby at the first pregnancy after examination, and four of five could have a baby, cumulatively. The patient with didelphys also succeeded at the first pregnancy after examination.

FLA 5.0 DTD ■ SEC CODE: RPL ■ fns24298 ■ 7 February 2009 ■ 3:45 pm ■ ce 11

TABLE 1

Baseline characteristics of patients with and without congenital uterine anomalies.

	Patients with anomalies (n = 42)	Patients without anomalies (n = 1528)	*P*
Maternal age, years			
Mean (SD)	31.5 (3.5)	31.1 (4.3)	NS
Median (interquartile range)	31 (29)	31 (28)	NS
Number of previous miscarriages			
2	17 (40.5)	765 (50.1)	
3	18 (42.9)	537 (35.1)	
4	7 (16.7)	136 (8.9)	
5 or more	0	90 (5.9)	.085
Mean (SD)	2.74 (0.77)	2.77 (1.12)	NS
Median (interquartile range)	3 (2)	2 (2)	NS
No. of previous live births			
0	37 (88.1)	1328 (86.9)	
1	4 (9.5)	186 (12.2)	
2 or more	1 (2.4)	14 (0.9)	NS
Mean (SD)	0.1	0.14 (0.37)	NS
No. of previous stillbirths			
0	40 (95.2)	1491 (97.6)	
One or more	2 (4.8)	37 (2.4)	NS

Note: Values are numbers (percentages) of patients unless otherwise specified.

Sugiura-Ogasawara. Uterine anomaly and recurrent miscarriage. Fertil Steril 2009.

Predictive Value for the D/C Ratio

Mean values (SD) for the D/C ratio in the miscarriage and live-birth groups were 0.8332 (0.3974) and 0.4776 (0.2745), respectively (P=.0057, 95% confidence interval [CI]: 0.1115–0.5998). When two miscarriage cases caused by an abnormal embryonic karyotype were excluded, the value for the D/C ratio in the miscarriage group was also significantly higher than in the live-birth group (P=.0051). Mean (SD) age and number of previous miscarriages for the 15 patients whose subsequent pregnancy ended in miscarriage and the 17 patients who experienced live births were 31.5 (3.0) versus 31.5 (3.8) and 2.76 (0.75) versus 2.72 (0.79), respectively (P = not significant). Ten patients were excluded because HSG films were not available.

The ROC curve is shown in Figure 1. From the figure, the cutoff value would be appropriate somewhere between 0.59 and 0.64, giving the sensitivity and specificity around 0.75–0.80. The area under the ROC curve, meaning the total diagnostic accuracy of the D/C ratio on live birth, was 0.808. From the logistic regression, the D/C ratio was found to be an independent risk factor on the failure of live birth after adjusting for age and previous number of miscarriages. The odds ratio for the 0.1 increment of D/C ratio was 1.42 (95% CI, 1.06–1.91).

DISCUSSION

In the present study, the live-birth rate of patients with congenital uterine anomalies tended to be lower, both at the first pregnancy after ascertainment and cumulatively, than that of patients with a normal uterus, although the differences were not significant. Congenital uterine anomalies were associated with miscarriages with a normal embryonic karyotype. Thus, congenital uterine anomalies impacted the progression of normal pregnancies.

Salim et al. earlier found no significant difference in the relative frequency of various anomalies or depth of fundal distortion as determined by three-dimensional (3D) ultrasound between women with and without a history of recurrent miscarriage, although abnormalities in uterine anatomy were more severe in women with a history of recurrent miscarriages (23). In this context, the finding in the present study that the D/C ratio is a predictor of further miscarriage in recurrent cases is clearly of interest.

However, 59.5% and 78.0% of our patients with a septate or bicornuate uterus without any kind of surgery could have a baby at the first pregnancy or cumulatively. Several studies concerning obstetric outcome after removal of a uterine septum have been reported (10–16). Lee et al. described a 77.3% uncomplicated delivery rate after hysteroscopic septum resection (14). Kormayos et al. compared pregnancy outcome after removal of septum between cases with and without a residual septum in patients with a history of two or three miscarriages and concluded that the live-birth rate in cases with no remnant was significantly higher than that in cases with a remnant (15). However, the live-birth rate for patients undergoing first hysteroscopy was 35.1% (33 of 94), and the

TABLE 2

Successful reproductive outcome after examination of uterine anomalies in patients with recurrent miscarriage.

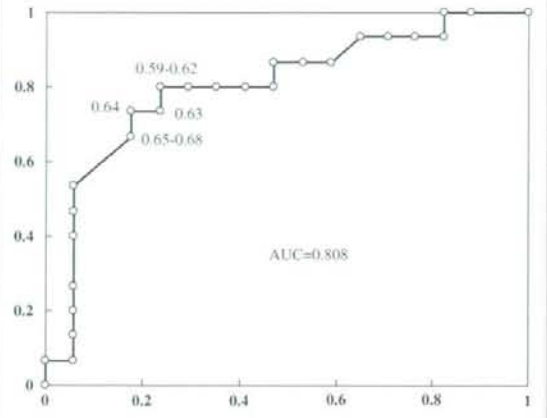
	Success rate per pregnancy						Cumulative success rate			
	With anomalies (n = 42)	Bicornuate	Septum	Without anomalies (n = 1528)	Difference in %	*P*	With anomalies (n = 41)[a]	Without anomalies (n = 1528)	Difference in %	*P*
Pregnancy after the ascertainment of uterine anomaly										
First	25/42 (59.5)[b]	21/37 (56.8)	4/5 (80.0)	1096/1528 (71.7)	−12.2	.084	25 (61.0)	1096 (71.7)	−10.7	.133
Second	5/9 (55.6)	4/8 (50.0)	2/2 (100)	166/275 (60.4)[c]	−4.8	.772	30 (73.2)	1262 (82.6)	−9.4	.119
Third	2/2 (100)	2/2 (100)		38/69 (55.0)	+45.0	.207	32 (78.0)	1300 (85.1)	−7.1	.215
Fourth				4/18 (22.2)				1304 (85.3)		
Fifth				3/9 (33.3)				1307 (85.5)		
Sixth				0/6 (0)				1307 (85.5)		
Final follow up							32 (78.0)	1307 (85.5)	−7.5	

Note: Values are numbers (percentages) of couples.
Success rate is defined as the live birth.
[a] One case underwent surgery between the first and second pregnancy after the ascertainment of an anomaly, thus this case was excluded from the cumulative analysis.
[b] Comparison was performed between patients both with anomalies and with normal uterus.
[c] Cases who could succeed in the first pregnancy were excluded from the analysis of the second and subsequent pregnancies.

Sugiura-Ogasawara. Uterine anomaly and recurrent miscarriage. Fertil Steril 2009.

FIGURE 1

ROC analysis of D/C ratio.



Sugiura-Ogasawara. Uterine anomaly and recurrent miscarriage. Fertil Steril 2009.

cumulative live-birth rate after one or two metroplasties was 54.3% (51 of 94). Both live-birth rates were lower than that without surgery in the present study. The benefits of surgical correction (open and hysteroscopic) on pregnancy outcome have yet to be assessed in a randomized trial, but the D/C ratio might be useful in deciding who should be selected.

Limitations

In the present study, clear uterine malformations such as septate, bicornuate, or unicornuate uterus and didelphys were found in 3.2% of patients. The prevalence of clear congenital uterine anomalies in patients with a history of recurrent miscarriages has been reported to be 1.8%–20.1% with the arcuate uterus excluded (5–7) and thus higher than the 2.2% documented for fertile women (28 of 1289) (2). Minor malformations like arcuate uterus do not appear to have any impact on reproduction (2), and therefore we here excluded cases with this anomaly.

HSG is the diagnostic modality that has most often led to a tentative diagnosis of congenital anomalies (19), but when used alone it cannot distinguish between a septate and a bicornuate uterus. Thus laparoscopy has hitherto been needed for a final diagnosis. The advent of sonohysterography, MRI (20), and 3D ultrasound now allows for accurate differential diagnosis (21), although distinguishing an arcuate from a mildly subseptate or bicornuate uterus still remains difficult.

It is important to distinguish between the bicornuate uterus and the septate uterus, especially regarding the selection of surgical methods because TCR should not be performed for the former. We here ascertained the type of anomaly to study the prevalence in accordance with the American Fertility Society classification of Mullerian anomalies. Woelfer et al. proved new 3D criteria by which a bicornuate uerus can be distinguished from a septate uterus when a fundal indentation >10 mm dividing the two cornua is detectable (21). Using 3D ultrasound, it has been found that the septate uterus has the higher incidence. The criteria are useful before deciding on using TCR for the septum. It is difficult to examine the significance of the distinction between bicornuate and septate uteri because of the absence of internationally established criteria, although we have given the live-birth rate for each anomaly in Table 2. Thus we focused not on type of anomalies but rather on the D/C ratio. In addition, the sample size in the anomaly group was too small to allow any conclusion when we distinguished between the two groups.

While we examined 1676 patients who became pregnant at least one time in the present study, we failed to follow up all those who received systemic examination for causes of recurrent miscarriage at our hospital because some lived at a long distance. Some patients might become infertile after miscarriage. A prospective case-control study should therefore be conducted to compare live-birth rates between patients with and without surgery, including consideration of the infertile rate.

Conclusion

Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage, being particularly associated with normal embryonic karyotype miscarriages. The height of the defect/length of the remaining uterine cavity ratio, the D/C ratio, has independent predictive value for further miscarriage in recurrent cases. Comparison of cases of anomalies with and without surgery is needed in future recurrent miscarriage studies.

REFERENCES

1. Farquharson RG, Pearson JF, John L. Lupus anticoagulant and pregnancy management. Lancet 1984;28:228–9.
2. Raga F, Bauset C, Remohi J, Bonilla-Musoles F, Simon C, Pellicer A. Reproductive impact of congenital Mullerian anomalies. Hum Reprod 1997;12:2277–81.
3. Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril 2000;73:300–4.
4. Sugiura-Ogasawara M, Ozaki Y, Sato T, Suzumori N, Suzumori K. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocation. Fertil Steril 2004;81:367–3.
5. Makino T, Umeuchi M, Nakada K, Nozawa S, Iizuka R. Incidence of congenital uterine anomalies in repeated reproductive wastage and prognosis for pregnancy after metroplasty. Int J Fertil 1992;37:167–70.
6. Clifford K, Rai R, Watson H, Regan L. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum Reprod 1994;9:1328–32.
7. Acien P. Incidence of Mullerian defects infertile and infertile women. Hum Reprod 1997;12:1372–6.
8. Jones HW, Jones GE. Double uterus as an etiological factor in repeated abortion: indications for surgical repair. Am J Obstet Gynecol 1953;65:325–39.
9. Strassmann EO. Operations for double uterus and endometrial atresia. Clin Obstet Gynecol 1961;4:240.

10. Goldenberg M, Sivan E, Sharabi Z, Mashiach S, Lipitz S, Seidman DS. Reproductive outcome following hysteroscopic management of intrauterine septum and adhesions. Hum Reprod 1995;10:2663–5.
11. Jacobsen LJ, DeCherney A. Results of conventional and hysteroscopic surgery. Hum Reprod 1997;12:1371–87.
12. Grimbizis G, Camus M, Clasen K, Tournaye H, De Munck L, Devroey P. Hysteroscopic septum resection in patients with recurrent abortions or infertility. Hum Reprod 1998;13:1188–93.
13. Karande VC, Gleicher N. Resection of uterine septum using gynaecoradiological techniques. Hum Reprod 1999;14:1226–9.
14. Lee R, Hickok LR. Hysteroscopic treatment of the uterine septum: a clinician's experience. Am J Obstet Gynecol 2000;182:1414–20.
15. Kormayos Z, Molar BG, Pal A. Removal of a residual portion of uterine septum in women of advanced reproductive age: obstetrics outcome. Hum Reprod 2006;21:1047–51.
16. Patton PE, Novy MJ, Lee DM, Hickok LR. The diagnosis and reproductive outcome after surgical treatment of the complete septate uterus, duplicated cervix and vaginal septum. Am J Obstet Gynecol 2004;190:1669–78.
17. Franssen MTM, Korevaar JC, van der Veen F, Leschot NJ, Bossuyt PMM, Goddijn M. Reproductive outcome after chromosome analysis in couples with two or more miscarriages: case-control study. Br Med J 2006;332:759–62.
18. Ogasawara M, Aoki K, Matsuura E, Sasa H, Yagami Y. Anti β2glycoprotein I antibodies and lupus anticoagulant in patients with recurrent pregnancy loss: prevalence and clinical significance. Lupus 1996;5:587–92.
19. American Fertility Society. The American Fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, Mullerian anomalies and intrauterine adhesions. Fertil Steril 1988;49:944–55.
20. Pellerito JS, McCarthy SM, Doyle MB, Glickman MG, DeCherney AH. Diagnosis of uterine anomalies: relative accuracy of MR imaging, endovaginal sonography, and hysterosalpingography. Radiology 1992;183:795–800.
21. Woelfer B, Salim R, Banerjee S, Elson J, Regan L, Jurkovic D. Reproductive outcomes in women with congenital uterine anomalies detected by three-dimensional ultrasound screening. Hum Reprod 2001;98:1099–103.
22. Tompkins P. Comments on the bicornuate uterus and twinning. Surg Clin N Am 1962;42:1049.
23. Salim R, Regan L, Woelfer B, Backos M, Jurkovic D. A comparative study of the morphology of congenital uterine anomalies in women with and without a history of recurrent first trimester miscarrige. Hum Reprod 2003;18:162–6.

1 **Midline uterine defect size is correlated with miscarriage of euploid embryos in recurrent cases**

M. S.-Ogasawara, Y. Ozaki, T. Kitaori, K. Kumagai, and S. Suzuki
Nagoya, Japan

Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage. Miscarriages in the setting of a resorption defect correlated with a higher loss of euploid embryos. The larger the defect, the greater the link with miscarriage.

The antigenic binding sites of autoantibodies to factor XII in patients with recurrent pregnancy losses

Akifumi Inomo*, Toshitaka Sugi*, Yoshimi Fujita, Hidehiko Matsubayashi, Shun-ichiro Izumi, Mikio Mikami
Department of Obstetrics and Gynecology, Specialized Clinical Science, Tokai University School of Medicine, Kanagawa, Japan

Summary
Recently, numerous studies have suggested an association between factor XII (FXII) deficiency and recurrent pregnancy losses, and between autoantibodies to FXII and recurrent pregnancy losses. Autoantibodies to FXII rather than FXII deficiency may be a risk factor for recurrent pregnancy losses. To know the pathogenesis of autoantibodies to FXII, epitope mapping study was done. Seventeen anti-FXII antibody positive recurrent pregnancy loss patients were chosen for this study. We used synthetic peptides in inhibition and direct binding studies to identify the antigenic binding site of autoantibodies to FXII. Among plasmas from 17 recurrent pregnancy loss patients who were positive for autoantibodies to FXII, 13 patients (76.5%) recognized amino acids 1–30, the amino-terminal heavy chain region that is known as factor XII binding site to platelet glycoprotein Ibα.

Keywords
Recurrent pregnancy losses, factor XII, kininogen, kallikrein-kinin system, antiphosphatidylethanolamine antibody, platelet

Thromb Haemost 2008; 99: 316–323

Introduction

Deficiencies of contact proteins such as factor XII (FXII) and high-molecular-weight kininogen are not associated with clinical bleeding despite marked prolonged activated partial thromboplastin time (aPTT), a surface-activated coagulation protein screening test. Paradoxically, studies suggest that contact proteins have anticoagulant, profibrinolytic functions in a physiologic milieu, on endothelial cells (1–6). Numerous clinical studies suggest that contact protein deficiencies may be associated with impaired contact factor-dependent fibrinolysis. This result may contribute to an increased incidence of thrombosis in patients with congenital FXII deficiency, an increased incidence of FXII deficiency in patients with venous thrombosis, and acquired thrombotic disorders such as myocardial infarction and re-thrombosis of coronary arteries after thrombolytic therapy (5–8). However, other studies suggest that FXII plays no role in ischemic vascular disease (9). Thus, it is unclear from existing literature whether a factor deficiency leads to thrombophilia.

Recently, numerous studies have suggested an association between contact protein deficiencies and recurrent pregnancy losses (10–12), and between autoantibodies to contact proteins and recurrent pregnancy losses (13, 14). Sugi and McIntyre (14) reported that certain antiphosphatidylethanolamine antibodies (aPE) are not specific for phosphatidylethanolamine (PE) *per se*, but are directed to PE-binding plasma proteins, kininogens. Sugi et al. tested recurrent pregnancy loss patients for aPE, especially those patients who lose during the embryonic period (<10 weeks' gestation). They showed a strong association between recurrent pregnancy loss and aPE, the latter of which requires the presence of kininogen or other plasma proteins (15, 16). In this study, 90.5% of the patients who were positive for plasma protein-dependent IgG aPE were kininogen-dependent. These data suggest that aPE may therefore represent a significant risk factor for early recurrent pregnancy loss.

Schved et al. (10) reported the cases of three young women with a FXII deficiency (two homozygous and one heterozygous) and a clinical history of spontaneous abortion. Braulke et al. (11) reported on eight patients with moderately reduced level of FXII found among 43 patients with repeated abortions. Recently, Gris et al. (12) reported the prevalence of haemostasis abnormalities in 500 unexplained primary recurrent aborters. They found 9.4% of the patients with an isolated FXII deficiency. Gallimore et al. (17) reported a high incidence (20.9%) of apparently true FXII deficiency in patients who were lupus anticoagulant (LA) positive. They have hypothesized that antibodies to FXII might be present in some patients who are LA positive and that immune complexes may be formed leading to reduced levels of FXII.

Correspondence to:
Toshitaka Sugi
Department of Obstetrics and Gynecology
Specialized Clinical Science, Tokai University School of Medicine
143 Shimokasuya, Isehara, Kanagawa, 259–1193, Japan
Tel.: +81 463 93 1121, Fax: +81 463 91 4343
E-mail: sugi@is.icc.u-tokai.ac.jp

*Both authors contributed equally to this report.

Received July 12, 2007
Accepted after major revision December 11, 2007

Prepublished online January 11, 2008
doi:10.1160/TH07-07-0447

They studied plasma samples from LA positive patients for the presence of antibodies to FXII and reported that many patients were positive for antibodies to FXII detected by ELISA and surface plasmon resonance (18). Jones et al. (19) reported that when levels of FXII were compared in patients with and without antibodies to FXII, significantly lower levels of FXII were seen in patients with antibodies to FXII. This suggests that the immune complex formation and subsequent sequestration resulted in reduced levels of FXII. They also reported that antibodies to FXII showed a strong and statistically significant association with recurrent fetal loss (odds ratio [OR] 5.4, p=0.025) (20). Autoantibodies to FXII rather than FXII deficiency may be a risk factor for thromboembolism and recurrent pregnancy losses.

In the present study, we used synthetic peptides in inhibition and direct binding studies to identify the antigenic binding sites of autoantibodies to FXII in patients with recurrent pregnancy losses.

Materials and methods

Sources of peptides

Peptides were synthesized at the Laboratory for Molecular Science Research, Tokai University School of Medicine. These peptides were synthesized on a PSSM-8 synthesizer (Shimadzu, Tokyo, Japan). The peptides were purified by preparative high performance liquid chromatography (HPLC) on a SynProPep cartridge system using reverse phase C_{18} columns (PepRPC18 column, Shimadzu). An amino acid sequence analysis was carried out using an automated protein/peptide sequencer, PPSQ-21 A system (Shimadzu). Nine overlapping and sequential peptides of 24–31 residues in length were synthesized to span the amino-terminal heavy chain region (Ile^{1}-Ala^{158}) of FXII: peptides IPP30, EPC30, PFQ30, HKC31, DQD30, HCS24, PCQ30, KCF31 and WYR28 (Table 1).

Patients

Blood samples were obtained with informed consent from 197 patients with recurrent pregnancy losses. Patients with recurrent pregnancy losses had two or more pregnancy losses before 10 weeks gestation, exclusive of ectopic pregnancy and/or elective abortion. All patients were evaluated by hysterosalpingography, vaginal ultrasound, karyotypes of both partners, endocrine monitoring (prolactin, thyroid function, fasting blood sugar, luteal phase progesterone), infectious factors (group B streptococcal and *Chlamydia trachomatis* infection), and autoantiboidies (lupus anticoagulant, anticardiolipin, antiphosphatidylserine, antiphosphatidylethanolamine, antinuclear antibodies, and anti-FXII antibodies). Anti-FXII antibodies were detected by the immunoblot. Seventeen previously determined anti-FXII antibody-positive patients with recurrent pregnancy loss were chosen for this study. The mean age of the patients was 34 years (range 26–41), and the mean number of pregnancy losses was 3.1 (range 2–4). Six, three and three of 17 patients each were positive for aPE, antiphosphatidylserine and anticardiolipin antibodies, respectively. Eight of 17 patients were negative for any antiphospholipid antibodies. No patients fulfilled criteria for definite antiphospholipid syndrome. FXII activities of 17 patients were 85.1 ± 38.3%. FXII activities of eight patients were less than 60%. All plasma samples were stored at –70°C until use.

SDS-PAGE and immunoblot

SDS-PAGE was done in 8% homogenous resolving gels and 3% stacking gels. Factor XIIa (Enzyme Research Laboratories, South Bend, IN, USA) was boiled for 2 minutes (min) in 2% SDS containing 0.1M Tris/Hcl, pH 6.8, 20% glycerol and 0.2% bromophenol blue. Transfer to nitrocellulose membrane was done overnight at 0.1 amps. Membranes were blocked for 1 hour (h) with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.3. Incubation with goat polyclonal antibodies (PoAb) to human factor XII (Enzyme Research Laboratories) or patient plasma (1/100) was done for 1 h followed by three washes with 0.03% Tween 20/PBS. The membrane was exposed to alkaline phosphatase conjugated antigoat immunoglobulins or monoclonal antibodies (MoAb) to human IgG for 1 h followed by washing as above. Using paranitrophenylphosphate buffer, the immunobands were developed.

Purification of IgG from plasmas

IgG was purified from plasmas by diethyl aminoethyl (DEAE)-Sepharose (Pharmacia LKB Biotechnology AB, Upsala, Sweden) followed by proein G-Sepharose (Pharmacia) chromatography. Plasma (1 ml) previously dialyzed in 20 mM Tris/HCl,

Table 1: Synthetic peptides derived from the amino-terminal heavy chain region of factor XII.

*Peptide	Sequence†	Positions‡
IPP30	IPPWEAPKEHKYKAEEHTVVLTVTGEPCHF	1~30
EPC30	EPCHFPFQYHRQLYHKCTHKGRPGPQPWCA	26~55
PFQ30	PFQYHRQLYHKCTHKGRPGPQPWCATTPNF	31~60
HKC31	HKCTHKGRPGPQPWCATTPNFDQDQRWGYCL	40~70
DQD30	DQDQRWGYCLEPKKVKDHCSKHSPCQKGGT	61~90
HCS24	HCSKHSPCQKGGTCVNMPSGPHCL	78~101
PCQ30	PCQKGGTCVNMPSGPHCLCPQHLTGNHCQK	84~113
KCF31	KCFEPQLLRFFHKNEIWYRTEQAAVARCQCK	115~145
WYR28	WYRTEQAAVARCQCKGPDAHCQRLASQA	131~158

*Peptides are identified by their three N-terminal residues using a one-letter code, followed by the total number of residues constituting the peptide. †A one-letter code for amino acid residues is used. ‡Relative positions of the plasma form of factor XII.

pH 7.4, was applied to the DEAE-Sepharose column (1x10 cm) equilibrated with 20 mM Tris/HCl, pH 7.4, and washed with the equilibration buffer at a flow rate of 20 ml/h. The effluent from the DEAE-Sepharose column was applied to a protein G-Sepharose column (5 ml) equilibrated with 20 mM Tris/HCl, pH 7.4. After washing with buffer, protein G-bound IgG was eluted with a 0.2 M glycine/HCl buffer, pH 3.0, and immediately neutralized with saturated Tris and dialyzed against Tris-buffered saline (TBS; 0.02 M Tris, 0.15 M NaCl, pH 7.3).

Mapping of the anti-FXII antibody epitope

Synthetic peptides covering the sequence of the amino-terminal heavy-chain region of FXII were examined for their reactivity with anti-FXII antibodies. Sumilon microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 µl of a 10 µM solution of the respective peptide in TBS. Each well was blocked for 1 h with 3% BSA in TBS containing 0.03% Tween 20 (Sigma) followed by incubation with 100 µl of anti-FXII antibody positive patient plasma diluted 1:100 or purified IgG (37.5 µg/ml) from patient plasma in 1% BSA/TBS containing 0.03% Tween 20 for 1 h. Alkaline phosphatase conjugated MoAb to human IgG (Sigma, St. Louis, MO, USA) was added, followed by the addition of substrate solution. The plates were washed three times with TBS containing 0.03% Tween 20 after peptide coating, blocking, serum and conjugate incubations. After color development produced by paranitrophenyl phosphate substrate, the optical density (OD) at 405 nm was measured. Color development was stopped with 75 µl of 3N NaOH when the OD of a positive control reached 1.0.

Inhibition of anti-FXII antibody binding to IPP30 by peptides

Sumilon plates were coated overnight at 4°C with 100 µl of a 10 µM solution of the IPP30 in TBS. Each well was blocked for 1 h with 3% BSA in TBS containing 0.03% Tween 20 (Sigma). Synthetic peptides IPP30 and HCS24 (1.5625, 3.125, 6.25, 12.5, 25, 50, or 100 µM) were incubated with 100 µl of anti-factor XII antibody-positive patient plasma (X) diluted 1:200 in 1% BSA/TBS containing 0.03% Tween 20 for 1 h. Alkaline phosphatase conjugated MoAb to human IgG (Sigma) was added followed by the substrate solution. The plates were washed three times with TBS containing 0.03% Tween 20 after peptide coating, blocking, serum and conjugate incubations. After color development produced by paranitrophenyl phosphate substrate, the OD at 405nm measured.

Results

Anti-FXII antibody detection by immunoblot

FXIIa was subjected to SDS-PAGE. The slab gel contents were transferred to nitrocellulose then immunoblotted with patient plasmas. All seventeen previously determined anti-FXII antibody positive recurrent pregnancy loss patients recognized FXIIa whole molecule under non-reducing conditions and many of them recognized heavy chain of FXII under reducing conditions (Fig. 1). No patient recognized the light chain of FXII.

Direct binding of anti-FXII antibodies to synthetic peptides

Nine overlapping and sequential peptides of 24–31 residues in length were synthesized to span the amino-terminal heavy chain region (Ile^{1}-Ala^{158}) of FXII: peptides IPP30, EPC30, PFQ30, HKC31, DQD30, HCS24, PCQ30, KCF31 and WYR28 (Table 1). These peptides cover the several structural domains, i.e. starting from the amino-terminus, a fibronectin domain type II, an epidermal growth factor-like domain, a fibronectin domain type I. These domains contain candidate sites for surface-binding regions or cell-binding regions of FXII such as amino acid

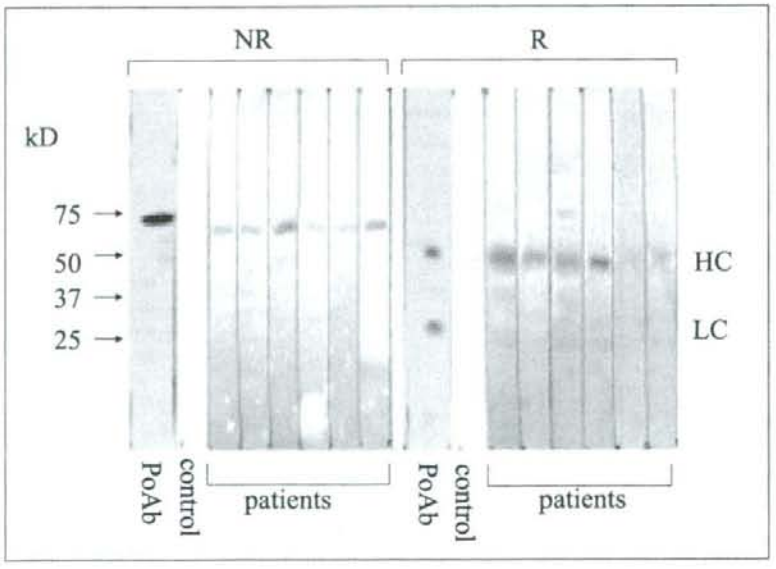


Figure 1: A representative experiment showing patient antibody binding to factor XIIa whole molecule and heavy chain of factor XII. Factor XIIa was subjected to SDS-PAGE under nonreducing (NR) and reducing (R) conditions. Immunoblots with polyclonal antibodies (PoAb) to FXII, normal plasma (control) or anti-factor XII antibody positive patient plasmas. HC: heavy chain, CL: light chain

Table 2: Binding of anti-factor XII antibodies to synthetic peptides in patients X. ELISA was performed using microtiter plates coated with 10 μM of the respective peptide. In control wells, no peptide was coated and only TBS was incubated. IgG anti-factor XII antibody-positive patient plasma X (1:100) was applied in triplicate wells followed by an alkaline phosphatase-conjugated secondary antibody. The absorption was measured at 405 nm. A plasma was considered positive if the difference between the OD in the ELISA with peptide and in the ELISA with control TBS was greater than 0.3.

IPP30	EPC30	PFQ30	HKC31	DQD30
1.064 ± 0.015	0.49 ± 0.009	0.473 ± 0.03	0.623 ± 0.013	0.493 ± 0.012
HCS24	**PCQ30**	**KCF31**	**WYR28**	**TBS**
0.411 ± 0.024	0.536 ± 0.024	0.457 ± 0.01	0.443 ± 0.005	0.45 ± 0.005

OD_{405nm} (mean ± SD).

residues 1–28, 39–47 and 134–153. These peptides were designed not to cause conformational changes in each domain by breaking internal disulfide loops. For example, peptide EPC30 (25–55) contains a fibronectin domain type II that has a disulfide bond between Cys^{28} and Cys^{54}. Likewise, HCS24 (78–101) covers the epidermal growth factor-like domain, which has a disulfide bond between Cys^{80} and Cys^{92}.

These nine peptides were examined for their reactivity with anti-FXII antibodies. ELISA was employed using microtiter plates coated with 10 μM of the respective peptide. IgG anti-factor XII antibody-positive patient plasma or normal control plasma (1:100) was applied followed by an alkaline phosphatase-conjugated secondary antibody. The absorption at 405 nm was measured. A plasma was considered positive if the difference between the OD in the ELISA with peptide and in the ELISA with control was greater than 0.3. This value was based on the evaluation of 41 normal individuals for direct binding to synthetic peptides by ELISA. From these data, the number of multiples of the median that accounted for 95% of this normal population ELISA values was 2.9. The OD associated with 2.9 multiples of the median was 0.234. To ensure the values were not borderline, 0.234 was rounded off to 0.3.

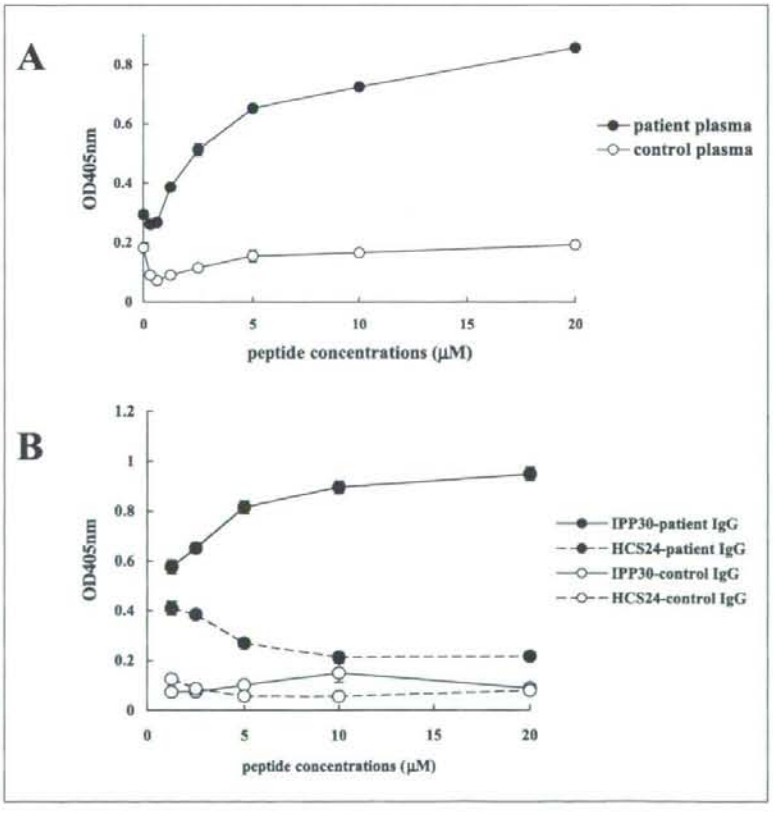


Figure 2: Binding of anti-factor XII antibodies to synthetic peptides. A) Increasing concentrations of synthetic peptide IPP30 was coated on microtiter plate wells. IgG anti-factor XII-positive patient plasma (X) or normal control plasma (1:100) was applied followed by an alkaline phosphatase-conjugated secondary antibody. The absorption at 405 nm was measured. B) Increasing concentrations of synthetic peptide IPP30 or HCS24 was coated on microtiter plate wells. Purified IgG (37.5 μg/ml) from patient X or normal control was applied followed by an alkaline phosphatase-conjugated secondary antibody. The absorption at 405 nm was measured.

Among the 17 recurrent pregnancy loss patients who were positive for anti-FXII antibodies, plasma from 13 patients (76.5%) recognized the IPP30 peptide, which encompasses the cell-binding sequences and surface-binding regions. Three patient plasmas (17.6%) recognized HKC31. Four patient plasmas (23.5%) recognized none of nine peptides.

Among 10 recurrent pregnancy loss patients who recognized only the IPP30 peptide, patient X was chosen for the dose-dependent and inhibition studies. This patient had a high titer of IgG anti-FXII antibody that recognized IPP30, but did not recognize eight other peptides (EPC30, PFQ30, HKC31, DQD30, HCS24,PCQ30, KCF31 and WYR28) (Table 2).

Binding of anti-FXII antibodies to synthetic peptide, IPP30

Increasing concentrations (0.3125, 0.625, 1.25, 2.5, 5, 10, 20 μM) of synthetic peptide IPP30 was coated onto microtiter plate wells. IgG anti-factor XII antibody-positive patient plasma (X), normal control plasma (1:100), purified IgG from patient X or normal control (37.5 μg/ml) was applied followed by an alkaline phosphatase-conjugated secondary antibody. The absorption at 405 nm was measured. As shown in Figure 2, anti-FXII antibodies bound to IPP30 in a concentration-dependent manner.

Synthetic peptide IPP30 (10 μM) was coated on microtiter plate wells. Several dilutions (1:6,400, 1:3,200, 1:1,600, 1:800, 1:400, 1:200, 1:100, 1:50) of IgG anti-FXII antibody-positive patient plasma (X) or normal control plasma were applied followed by an alkaline phosphatase-conjugated secondary antibody. Likewise, several concentrations of purified IgG from patient X or normal control were applied followed by a secondary antibody. Absorption at 405 nm was measured. Anti-FXII antibodies were observed to bind to IPP30 in a concentration-dependent manner (Fig. 3).

Inhibition of anti-FXII antibody binding to IPP30 by peptides

The plasma from a IgG anti-FXII antibody-positive patient (X) (1:200) was incubated with increasing concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50, 100 μM) of synthetic peptide IPP30 or control peptide, HCS24 in microtiter plate wells that were coated with 10 μM of the IPP30. As shown in Figure 4A, IPP30 blocked anti-FXII antibody binding to IPP30 in a concentration-dependent manner.

Purified IgG (37.5 μg/ml) from patient X or normal control was incubated with synthetic peptide IPP30 (50 μM) in microtiter plate wells that were coated with 10 μM of the IPP30. As shown in Figure 4B, IPP30 blocked anti-factor XII antibody binding to IPP30.

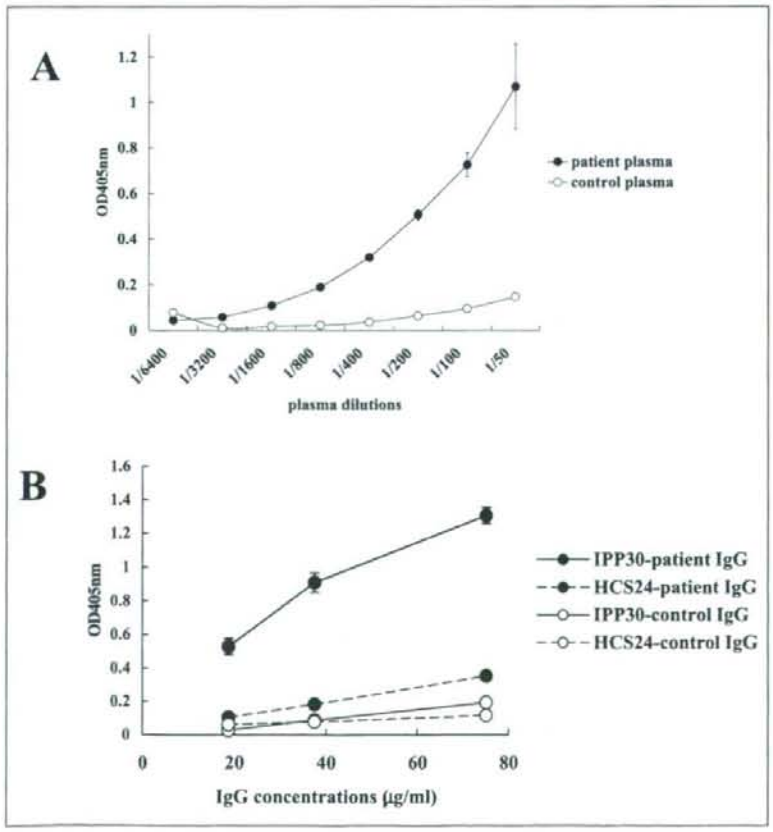


Figure 3: Dose-dependent anti-factor XII antibody-binding to synthetic peptides. A) Synthetic peptide IPP30 (10 μM) was coated on microtiter plate wells. Several dilutions of IgG anti-factor XII antibody-positive patient plasma (X) or normal control plasma were applied followed by an alkaline phosphatase-conjugated secondary antibody. The absorption at 405nm was measured. B) Synthetic peptide IPP30 or HCS24 (10 μM) was coated on microtiter plate wells. Several concentrations of purified IgG from patient X or normal control were applied followed by an alkaline phosphatase-conjugated secondary antibody. The absorption at 405 nm was measured.

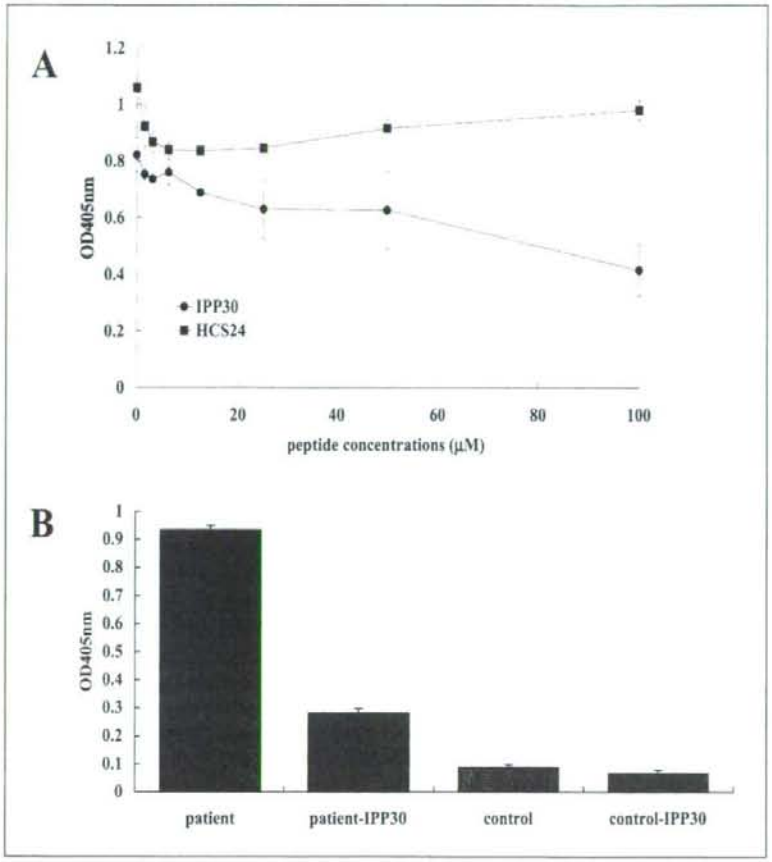


Figure 4: Inhibition of anti-factor XII antibody-binding to IPP30 by peptides.
A) IgG anti-factor XII antibody-positive patient (X) plasma (1:200) was incubated with increasing concentrations of synthetic peptide IPP30 or HCS24 as control in microtiter plate wells that were coated with 10 μM of the IPP30. B) Purified IgG (37.5 μg/ml) from patient X or normal control was incubated with synthetic peptide IPP30 (50 μM) in microtiter plate wells that were coated with 10 μM of the IPP30.

Discussion

Although some studies have identified FXII deficiency as a risk factor for recurrent pregnancy loss (10–12), others failed to find such a relationship (21). Recently, Pauer et al. generated mice deficient for FXII using a gene targeting approach (22). Homozygous FXII-knockout mice showed no FXII plasma activity and had a markedly prolonged aPTT. Interestingly, they reported that matings of $FXII^{-/-}$ males and $FXII^{-/-}$ females resulted in normal litter sizes demonstrating that total FXII deficiency in $FXII^{-/-}$ females does not affect pregnancy outcome (22). Iwaki et al. also reported that in female mice homozygous for a total FXII deficiency, normal deliveries occurred with normal litter sizes (23). In contrast, Jones et al. reported that antibodies to FXII showed a strong and statistical significant association with recurrent fetal loss (OR 5.4, p=0.025) (20). They reported that when levels of FXII were compared in patients with and without antibodies to FXII, significantly lower levels of factor XII were seen in patients with antibodies to FXII (19). This suggests that the immune complex formation and subsequent sequestration resulted in reduced levels of FXII. Autoantibodies to FXII rather than FXII deficiency may be a real risk factor for recurrent pregnancy losses.

HK inhibits thrombin-induced platelet aggregation by inhibiting the binding of thrombin to platelets (24). Domain 3 of HK is responsible (25). Recently, the binding site on platelets, which mediates this effect, was shown to be glycoprotein (GP) Ib-IX complex (26). It has been reported that HK and FXII compete for the same binding site on endothelial cells (27). Bradford et al. reported that FXIIa also inhibits thrombin interaction with platelets in a mechanism also involving binding to the same receptor (28). HK and FXII both directly bind to glycocalicin, the extra cellular subunit of GP Ibα, in a Zn^{2+}-dependent manner. They also reported that FXII binding to platelets was inhibited by monoclonal antibody B7C9, whose non-contiguous epitopes have been mapped to amino acids 1–28 and an icosapeptide in the "finger region" of FXII (29). Interestingly, our present study demonstrates that among plasmas from 17 recurrent pregnancy loss patients who were positive for autoantibodies to FXII, 13 patients (76.5%) recognized amino acids 1–30. This suggests that autoantibodies to FXII in patients with recurrent pregnancy losses may inhibit FXII binding to platelets and may cause pregnancy loss.

The kininogens can inhibit platelet aggregation induced by thrombin. Domain 3 of the kininogen heavy chain was found to inhibit thrombin from binding to the platelet thrombin receptor.

By using specific monoclonal antibodies, Jiang et al. showed that it is the domain 3 region that is responsible for the inhibition of thrombin binding to platelets (25). Kunapuli et al. found that recombinant domain 3 inhibited thrombin-induced platelet aggregation (30). Sugi and McIntyre (14) reported that certain aPE are not specific for PE *per se*, but are directed to PE-binding plasma proteins, kininogens. Several studies report strong association of aPE with thrombosis and recurrent pregnancy losses (15, 16, 31). Sugi and McIntyre hypothesized that when bound by aPE, the platelet-kininogen complex may no longer render the platelet refractory to thrombin activation, thus predisposing to aggregation and thrombosis. Their in-vitro data (32) support these observations as they demonstrated that kininogen-dependent IgG-aPE purified from several aPE-positive patient plasmas caused marked augmentation of thrombin-induced platelet aggregation, but did not affect ADP-induced platelet aggregation. Moreover, kininogen-independent IgG-aPE did not affect thrombin-induced platelet aggregation. For this to occur, it is possible that aPE may recognize the domain 3 region of kininogens subsequent to their binding platelet. Herwald et al. (33) reported that a monoclonal antibody to domain 3, HKH15, which interferes with the complex formation between kininogen and papain, also blocked the cell binding of kininogens and was directed to the extreme carboxyl-terminal portion of domain 3. The epitope of HKH15, which binds to domain 3 and blocks the binding of kininogens to platelets and endothelial cells, was mapped using synthetic peptides, which span the entire domain 3 sequence. They reported that one peptide, LDC27, specifically bound to HKH15. Fine mapping of the epitope of HKH15 has also revealed a minimal 13-residue segment in LDC27, named CNA13, to be the antibody-binding site. Katsunuma et al. (34) reported that among plasmas from 24 recurrent pregnancy loss patients who were positive for kininogen-dependent IgG-aPE, 17 (70.8%) recognized the LDC27 peptide. They mapped the aPE-binding region to domain 3 using a plasma specimen from a patient with recurrent pregnancy loss. Interestingly, the aPE of a patient recognized CNA13, which is identical to the epitope of HKH15. Leu331-Met357 (LDC27) and Cys333-Lys345 (CNA13) are located on the carboxyl-terminal portion of kininogen domain 3, which is known as the major kininogen heavy-chain cell attachment site where it overlaps its cysteine protease inhibitory region. Because aPE interferes with the balance of haemostasis *in vitro*, aPE may therefore induce a similar condition in patients thereby causing thrombosis and recurrent pregnancy losses.

Many patients with recurrent pregnancy losses have both FXII deficiency and aPE. In FXII-deficient patients with recurrent pregnancy losses, 32.4% were positive for aPE (T. Sugi, unpublished data). From our epitope mapping studies, both autoantibodies to FXII and kininogen-dependent aPE may block FXII- and kininogen-biding to GP Ib-IX-V complex and augment thrombin-induced platelet aggregation. Thus autoantibodies to FXII and kininogens may cause thrombosis and recurrent pregnancy losses.

Recently, Harris et al. reported the antigenic binding site(s) of antibodies to FXII associated with the antiphospholipid syndrome (35). They investigated plasma samples from 12 female patients with definite antiphospholipid syndrome for the presence of antibodies to FXII. To investigate the antigenic binding site(s) of FXII, 150 peptides of the known FXII sequence were synthesized. Seven patients positive for FXII antibodies were chosen, and each patient's purified IgG or IgM was tested against each peptide. Plasma from only one of the seven patients showed binding to the synthetic peptides. In this patient, two regions were identified as possible antigenic binding site(s) for FXII antibodies: one in the growth factor domain and the other in the catalytic domain. There was no convincing explanation how these antibodies may inhibit the physiological function of FXII and contribute to the clinical symptoms suffered by this patient group. In our present study, we tested the antigenic binding site(s) of antibodies to FXII in patients with recurrent pregnancy losses. A difference between their study and our present study is that no patients studied fulfilled criteria for definite antiphospholipid syndrome in our study.

In conclusion, autoantibodies to FXII in patients with recurrent pregnancy losses recognize amino-terminal heavy chain region of FXII that is known as FXII-binding site to platelet glycoprotein Ibα. This suggests that aoutoantibodies to FXII in patients with recurrent pregnancy losses may inhibit the physiological role of FXII and thus contribute to the pregnancy loss.

Acknowledgements

Support from the Japan Society for the Promotion of Science to T. Sugi (#18591815) is gratefully acknowledged.

References

1. Colman RW, Schmaier AH. Contact system: A vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. Blood 1997; 90: 3819–3843.

2. Wuepper KD, Miller DR, Lacombe MJ. Flaujeac trait. Deficiency of human plasma kininogen. J Clin Invest 1975; 56: 1663–1672.

3. Saito H, Ratnoff OD, Waldmann R, et al. Fitzgerald trait: Deficiency of a hitherto unrecognized agent, Fitzgerald factor, participating in surface mediated reactions of clotting, fibrinolysis, generation of kinins, and the property of diluted plasma enhancing vascular permeability (PF/DIL). J Clin Invest 1975; 55: 1082.

4. Halbmayer WM, Haushofer A, Schon A, et al. The prevalence of moderate and severe factor XII (Hageman factor) deficiency among the normal population: Evaluation of the incidence of factor XII deficiency among 300 healthy blood donors. Thromb Haemost 1994; 71: 68–72.

5. Lammle B, Wuillemin WA, Huber I, et al. Thromboembolism and bleeding tendency in congenital factor XII deficiency-A study on 74 subjects from 14 swiss families. Thromb Haemost 1991; 65: 117–121.

6. Halbmayer WM, Mannhalter C, Feichtinger C, et al. The prevalence of factor XII deficiency in 103 orally anticoagulated outpatients suffering from recurrent venous and/or arterial thromboembolism. Thromb Haemost 1992; 68: 285–290.

7. Mannhalter C, Fischer M, Hopmeier P, et al. Factor XII activity and antigen concentrations in patients suffering from recurrent thrombosis. Fibrinolysis 1987; 1: 259–263.

8. Jespersen J, Munkvad S, Pedersen OD, et al. Evidence for a role of factor XII-dependent fibrinolysis in cardiovascular diseases. Ann NY Acad Sci 1992; 667: 454.

9. Oguchi S, Ito D, Murata M, et al. Genotype distribution of the 46C/T polymorphism of coagulation factor XII in the Japanese population: absence of its association with ischemic cerebrovascular disease. Thromb Haemost 2000; 83: 178–179.

10. Schved JF, Dupaigne D, Gris JC, et al. Factor XII congenital deficiency and early spontaneous abortion. Fertil Steril 1989; 52: 335–336.

11. Braulke I, Hinney B, Pruggmayer M, et al. Factor XII (Hageman) deficiency in women with habitual abortion: new subpopulation of recurrent aborters? Fertil Steril 1993; 59: 98–101.

12. Gris JC, Neveu SR, Maugard C, et al. Prospective evaluation of the prevalence of haemostasis abnormalities in unexplained primary early recurrent miscarriages. Thromb Haemost 1997; 77: 1096–1103.
13. McIntyre JA, Wagenknecht DR, Sugi T. Phospholipid binding plasma proteins required for antiphospholipid antibody detection-an overview. Am J Reprod Immunol 1997; 37: 101–110.
14. Sugi T, McIntyre JA. Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen-PE complex. Blood 1995; 86: 3083–3089.
15. Sugi T, Katsunuma J, Izumi S, et al. Prevalence and heterogeneity of antiphosphatidylethanolamine antibodies in patients with recurrent early pregnancy losses. Fertil Steril 1999; 71: 1060–1065.
16. Sugi T, Matsubayashi H, Inomo A, et al. Antiphosphatidylethanolamine antibodies in recurrent early pregnancy loss and mid-to-late pregnancy loss. J Obstet Gynaecol Res 2004; 30: 326–332.
17. Gallimore MJ, Jones DW, Winter M. Factor XII determinations in the presence and absence of phospholipid antibodies. Thromb Haemost 1998; 79: 87–90.
18. Jones DW, Gallimore MJ, Harris S, et al. Antibodies to FXII associated with lupus anticoagulant. Thromb Haemost 1999; 81: 387–390.
19. Jones DW, Gallimore MJ, Mackie IJ, et al. Reduced factor XII levels in patients with the antiphospholipid syndrome are associated with antibodies to factor XII. Br J Haematol 2000; 110: 721–726.
20. Jones DW, Mackie IJ, Gallimore MJ, et al. Antibodies to factor XII and recurrent fetal loss in patients with the anti-phospholipid syndrome. Br J Haematol 2001; 113: 550–552.
21. Matsuura T, Kobayashi T, Asahina T, et al. Is factor XII deficiency related to recurrent miscarriage? Semin Thromb haemost 2001; 27: 115–120.
22. Pauer HU, Renne T, Hemmerlein B, et al. Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo. Thromb Haemost 2004; 92: 503–8.
23. Iwaki T, Castellino FJ. Plasma levels of bradykinin are suppressed in factor XII-deficient mice. Thromb Haemost 2006; 95: 1003–1010.
24. Puri RN, Zhou F, Hu CJ, et al. High molecular weight kininogen inhibits thrombin-induced platelet aggregation and cleavage of aggregin by inhibiting binding of thrombin to platelets. Blood 1991; 77: 500–507.
25. Jiang YP, Muller-Esterl W, Schmaier AH. Domain 3 of kininogens contains a cell-binding site and a site that modifies thrombin activation of platelets. J Biol Chem 1992; 267: 3712–3717.
26. Bradford HN, Dela Cadena RA, Kunapuli SP, et al. Human kininogens regulate thrombin binding to platelets through the glycoprotein Ib-IX-V complex. Blood 1997; 90: 1508–1515.
27. Reddigari SR, Shibayama Y, Brunnee T, et al. Human Hageman factor (factor XII) and high molecular weight kininogen compete for the same binding site on human umbilical vein endothelial cells. J Biol Chem 1993; 268: 11982–11987.
28. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J Biol Chem 2000; 275: 22756–22763.
29. Clarke BJ, Cote HCF, Cool DE, et al. Mapping of a putative surface-binding site for human coagulation factor XII. J Biol Chem 1989; 264: 11497–11502.
30. Kunapuli SP, Bradford HN, Jameson BA, et al. Thrombin-induced platelet aggregation is inhibited by the heptapeptide Leu271-Ala277 domain 3 in the heavy chain of high molecular weight kininogen. J Biol Chem 1996; 271: 11228–11234.
31. Sanmarco M, Alessi MC, Harle JR, et al. Antibodies to phosphatidylethanolamine as the only antiphospholipid antibodies found in patients with unexplained thromboses. Thromb Haemost 2001; 85: 800–805.
32. Sugi T, McIntyre JA. Autoantibodies to kininogen-phosphatidylethanolamine complexes augment thrombin-induced platelet aggregation. Thromb Res 1996; 84: 97–109.
33. Herwald H, Hasan AAK, Godovac-Zimmermann J, et al. Identification of an endothelial cell binding site on kininogen domain 3. J Biol Chem 1995; 270: 14634–14642.
34. Katsunuma J, Sugi T, Inomo A, et al. Kininogen domain 3 contains regions recognized by antiphosphatidylethanolamine antibodies. J Thromb Haemost 2003; 1: 132–138.
35. Harris SL, Jones DW, Gallimore MJ, et al. The antigenic binding site(s) of antibodies to factor XII associated with the antiphospholipid syndrome. J Thromb Haemost 2005; 3: 969–975.

The American Journal of Pathology, Vol. 173, No. 3, September 2008

DOI: 10.2353/ajpath.2008.071169

Cell Injury, Repair, Aging and Apoptosis

Granulysin Produced by Uterine Natural Killer Cells Induces Apoptosis of Extravillous Trophoblasts in Spontaneous Abortion

Akitoshi Nakashima,* Arihiro Shiozaki,* Subaru Myojo,* Mika Ito,* Mikiko Tatematsu,* Masatoshi Sakai,* Yasushi Takamori,† Kazuyuki Ogawa,‡ Kinya Nagata,‡ and Shigeru Saito*§

From the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Toyama, Toyama; the StemCell Institute Inc.,† Tokyo; the R&D Center,‡ BML, Saitama; and the 21st Century COE Program,§ University of Toyama, Toyama, Japan*

Immune changes are known to occur in recurrent spontaneous abortion, but it is unclear whether either maternal natural killer (NK) cells or T cells attack fetus-derived trophoblasts. To clarify the immunological causes of spontaneous abortion, we examined the relationship between cytotoxic granule proteins in decidual lymphocytes, such as granulysin, granzyme B, and perforin, and the induction of apoptosis in extravillous trophoblasts (EVTs). The number of granulysin-positive CD56^{bright} NK cells increased significantly in the decidua basalis during spontaneous abortion compared with normal pregnancy; however, granzyme B- and perforin-positive cells did not change. Interestingly, the expression of granulysin was also detected in the nuclei of EVTs in spontaneous abortion samples. When IL-2-stimulated CD56^{bright} NK cells were cocultured with EVT cells (HTR-8/SV40neo), granulysin was found initially in the cytoplasm and then accumulated in the nuclei of the HTR-8/SV40neo cells. Furthermore, transfected cells expressing a GFP-granulysin fusion protein induced apoptosis in HTR-8/SV40neo cells independently of caspases. Our results suggest that granulysin-positive uterine NK cells attack EVTs; subsequently, the uNK-derived granulysin actively accumulates in the nuclei of EVTs, causing the death of EVTs due to apoptosis. These data support a new apoptosis pathway for trophoblasts via uNK-derived granulysin, suggesting that granulysin is involved in spontaneous abortion. *(Am J Pathol 2008, 173:653–664; DOI: 10.2353/ajpath.2008.071169)*

Apoptosis in trophoblasts is well known to be involved in human spontaneous abortion, intrauterine growth restriction, and preeclampsia.[1,2] However, direct evidence that cytotoxic T cells or natural killer (NK) cells cause apoptosis in extravillous trophoblasts (EVTs) has not been reported in humans, and little is known about the precise mechanism of apoptosis in EVTs of spontaneous abortion. Namba et al showed that interleukin (IL)-2Rβ (CD122)-overexpressing transgenic mice showed 100% viable fetuses at gestational day 8 but 100% fetal death at gestational day 12, and proposed that excessive activation of uterine NK (uNK) cells can cause abortion.[3] In these mice, uNK cells were abnormally found within the placenta and the trophoblast layers had lost continuity.

Decidualization of the human endometrium following embryo implantation is normally associated with massive recruitment of CD16$^-$CD56^{bright} NK cells. In early pregnant decidua, CD16$^-$CD56^{bright} NK cells constitute the major immune cell population accounting for more than 70% among lymphocytes, whereas CD4$^+$ T cells and CD8$^+$ T cells are a minor population (<5%).[4,5] NK cells interact with target cells by a series of inhibitory and activating NK cell receptors constitutively expressed by uNK cells. Furthermore, CD16$^-$CD56^{bright} NK cells recognize the major histocompatibility complex class I molecules HLA-C, -E, and -G via these inhibitory and activating receptors.[6] Once balance is lost, excessive activation signals may induce trophoblast lysis by these activating NK cells.[2,7] Hyperactivated NK cells release cytolytic granules such as granzymes and granulysin through perforin-induced pores. The distribution of perforin-positive uNK cells is essentially the same between

Supported by Grants-in-Aid for Scientific Research (B)–17390447 and (C)–18591797, Grant-in-Aid for Exploratory Research 18659482, and Grant-in-Aid for Young Scientists (B)–19791139 from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the 21st Century COE Program.

A.N. and A.S. contributed equally to this work.

Accepted for publication June 12, 2008.

Address reprint requests to Shigeru Saito, M.D., Ph.D., Department of Obstetrics and Gynecology, Faculty of Medicine, University of Toyama, 2630 Sugitani, Toyama, 930-0194, Japan. E-mail: s30saito@med.u-toyama.ac.jp.

normal and abortion model mice,[8] although a slight elevation of perforin-positive uNK cells in human sporadic miscarriage with a normal fetal chromosomal karyotype has been reported.[9] On the other hand, the distribution of granulysin-positive lymphocytes in normal pregnancy and spontaneous abortion cases has not been reported.

Granulysin is a novel cationic molecule present in the granules of cytotoxic T cells and NK cells.[10–12] Two molecular forms of granulysin result from post-translational cleavage at both the amino and carboxyl termini. The protein is synthesized as a 15-kd precursor form, which is sorted to the cytolytic granules where it is processed into a 9-kd effector form.[10,13] Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including tumor cells, transplant cells, bacteria, fungi, and parasites,[11–15] damaging negatively charged cell membranes because of its positive charge.[16] Granulysin is coexpressed with and functionally related to both perforin, a pore-forming protein related to the membrane attack complex of the complement, but structurally distinct, and granzymes, serine esterases that induce apoptosis by activating caspases.[17] Thus, granulysin plays important roles in the host defense against pathogens, and also induces apoptosis of the target cells in a mechanism involving caspases and other pathways.[16,18]

There is, so far, no report about the relationship between apoptosis of trophoblasts and uterine NK cells including granulysin in spontaneous abortion. We hypothesized that uNK cells contact and lyse EVTs, which detach from cytotrophoblast cell column by the release of cytotoxic granule proteins in decidua. In this study, we show that uNK cells induce apoptosis of EVTs in a granulysin-dependent manner in spontaneous abortion cases.

Materials and Methods

Tissue Collection

All samples for this study were approved by University of Toyama Ethics Committee, and informed consent was obtained from all patients. Ten specimens from elective termination of pregnancy (maternal age median 29 years, range 21–35 years; gestational age median 8 weeks, range 6–10 weeks) were obtained. These specimens were treated as normal pregnant subjects. Gestational age was calculated from the last menstrual period and confirmed by ultrasound measurements of crown-rump length. Twenty samples from first-trimester spontaneous abortion (maternal age median 30 years, range, 20–41 years; gestational age median 8 weeks, range 6–11 weeks) were collected. Anembryonic pregnancies or fetal death was confirmed by ultrasonography. All samples were collected by vaginal curettage; in normal pregnancy and spontaneous abortion, curettage was carried out within 24 hours after diagnosis. Both groups received the same exclusionary criteria: women receiving any medication or with infectious, autoimmune, or other systemic or local diseases were excluded. Clinical details were recorded for each woman (Table 1). Karyotype analysis was not performed in the spontaneous abortive specimens. The tissue samples were fixed in formalin and embedded in paraffin blocks for histological examination and immunohistochemical staining.

Table 1 Comparison of Clinical Data from Patients with Spontaneous Abortions and Controls

	Normal control (n = 10)	Spontaneous abortion (n = 20)
Age (year)	29 (21–35)	30 (20–41)
Gravidity[a]	1 (0–4)	1 (0–3)
PH: spontaneous abortion[b]	n = 1	n = 2
Gestational weeks	8 (6–10)	8 (6–11)

Data are expressed as median (range).
[a]Abotrion times and live birth times.
[b]Numbers of patients with spontaneous abortion in past history (PH), excluding the abortion of this study.

Isolation of Decidual Lymphocytes

Samples of decidua from different patients were not mixed to avoid the induction of allogenic reaction of leukocytes. For isolation of decidual cells, specimens from decidua of spontaneous abortion or normal pregnancy (gestational age, 6–10 weeks) were dissected free of products of conception and washed twice in phosphate-buffered saline (PBS). The total decidua tissue (4–9 g) was then minced into fragments of ~1 mm^3 and digested for 20 minutes at 37°C under slight agitation in Dulbecco's modified Eagle's medium containing 0.125% trypsin (Sigma), 4.2 mmol/L $MgSO_4$, 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 50 Kunitz U/ml deoxyribonuclease type IV (Sigma). The cell suspension obtained was filtered through sterile stainless steel 50-μm wire mesh and washed once in PBS. The decidual mononuclear cells (leukocytes) were purified by the standard Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) density gradient centrifugation after homogenization and filtration through a 32-mm nylon mesh as previously reported.[4] The cells were then suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated for 2 hours at 37°C in an atmosphere of 5% CO_2 to allow adherent cells to attach to the plastic. The supernatant containing decidual lymphocytes was then collected and the cells were used for analysis.

Cell Lines and Transfection

HTR-8/SV40neo cells (a kind gift from Dr. Charles H. Graham, Department of Anatomy and Cell Biology, Queen's University, Ontario, Canada), an EVT cell line, JEG-3 and BeWo, choriocarcinoma cell lines, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. JAR choriocarcinoma cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. For transient expression of GFP-fused protein, 3- to 7 × 10^5 cells were inoculated into a 30-mm glass

base dish (Asahi Techno Glass, Tokyo, Japan) or a six-well plate (Becton Dickinson, San Jose, CA) a day before transfection. HTR-8/SV40neo cells were transfected with 1 μg of each plasmid DNA using FuGENE 6 reagent (Roche Diagnostics, Basel, Switzerland) and cultured for the times indicated. On the other hand, transient transfection of JAR and JEG-3 cells was performed with Lipofectamine LTX reagent (Invitrogen Corp., Carlsbad, CA). After that, the cells were analyzed by flow cytometry or immunocytochemistry.

For the assay of granulysin localization, we counted the number of the cells in which GFP completely merged with Hoechst 33342 nuclear staining as nuclear-localized cells, and the other cells as nuclear and cytoplasm-localized cells, respectively, in 100 GFP-positive cells. Each experiment was performed at least three times.

Plasmid Construction

A GFP-expression vector, GFP-granulysin, was previously constructed.[19] Additionally, to construct GFPx2-fused proteins, cDNAs for a 9-kd granulysin corresponding to amino acid sequence G^{63} through R^{136} of the full-length granulysin were amplified by reverse transcription-polymerase chain reaction with Pfu polymerase (Stratagene) using total mRNA from normal peripheral blood mononuclear cells as a template. An expression vector, which expresses tandemly arranged GFP, was kindly provided by Dr. Naoko Imamoto (Discovery Research Institute, RIKEN, Saitama, Japan). The PCR product was cloned into the *Hin*dIII/*Bam*HI sites at the C′ ends of GFP (Figure 1).

Immunohistochemistry

Five-micron sections from formalin-fixed, paraffin-embedded human tissues were deparaffinized in xylene and rehydrated in a graded series of alcohol followed by antigen retrieval by boiling in citrate buffer at 121°C for 15 minutes in an autoclave. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol for 15 minutes, and nonspecific binding was blocked by incubating the sections in 5% normal goat serum. After extensive washes with PBS, the sections were reacted with a biotin-labeled anti-granulysin mouse monoclonal antibody (mAb) (1:100, RC8)[20] and mouse monoclonal antibody anti-CD56 (NCAM) (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Further processing of the sections for the detection was performed according to the manufacturer's instructions provided with the Vectastain kit (Vector Laboratories, Burlingame, CA) or Vector blue alkaline phosphatase substrate kit (Vector Laboratories). After washing, sections were counterstained with Mayer's hematoxylin, washed in water, and successively immersed in graded ethanol solutions and xylene before coverslipping. In control sections, the primary antibody was replaced by control nonimmune mouse IgG (Vector Laboratories). Other first antibodies were used as follows: anti-perforin mAb (1:200, Thermo Scientific, Fremont, CA), anti-granzyme B mAb (1:200, Santa Cruz Biotechnology) and anti-cytokeratin mAb (AE1/AE2, 1:250, DAKO, Carpinteria, CA). When counting the percentage of positive cells in tissues, at least five high power fields were chosen randomly. Confocal images of fluorescent materials in spontaneous abortive tissues were collected using a confocal laser scanning microscope model TCS-SP5 (Leica Microsystems, Wetzler, Germany). For immunohistochemical fluorescent samples, the Alexa Fluor 488 goat anti-mouse IgG antibody and the streptavidin, Alexa Fluor 594 conjugate antibody, were used as secondary antibodies (Molecular Probes Inc., Eugene, OR).

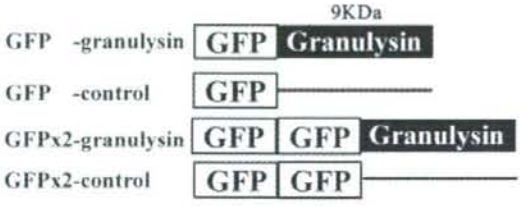


Figure 1. Structure of granulysin expression vectors. The structures of GFP-fused granulysin expression vectors are shown.

Immunocytochemistry

The cells were fixed at 4% paraformaldehyde-PBS for 15 minutes and labeled with the first antibody, biotin-labeled anti-granulysin antibody (RC8) or anti-cleaved cytokeratin 18 mAb at dilution of 1:100. Negative controls were performed by replacement of the primary antibody with normal mouse serum. They were secondarily stained with the Alexa Fluor 594-streptavidin or the Alexa Fluor 594-conjugated anti-mouse IgG (Molecular Probes), respectively. They were finally stained with Hoechst 33342 (H33342) (Sigma-Aldrich, St. Louis, MO) for 10 minutes, washed with PBS, and observed under an All-in-One type fluorescence microscope BZ-8000 (KEYENCE, Osaka, Japan).

Purification of CD56$^+$ Cells, Transwell Experiments, and Coculture of HTR-8/SV40neo with Decidual Lymphocytes

Isolated decidual lymphocytes were stimulated with or without 1 ng/ml IL-2 for 24 hours and then were gently washed with PBS three times. A small portion of the cells was fixed on a plate to check granulysin expression. For the isolation of CD56$^+$ lymphocytes, decidual lymphocytes were incubated with anti-CD56 mAb (Becton Dickinson) at 4°C for 20 minutes and then incubated with magnetic anti-mouse IgG beads. CD56$^+$ cells were separated using a magnetic cell sorting column. Flow cytometric analysis revealed that the purity of CD56$^+$ cells was >95%. These cells (1×10^7) were added directly to 1×10^6 HTR-8/SV40neo for 24 hours. Subsequently, these cells were washed with PBS three times to remove the decidual lymphocytes and then were observed after fixation. Alternatively, to inhibit cell-cell contact, decidual lymphocytes were placed in a 0.2-μm Anopore membrane Nunc culture insert (Nalge Nunc International) and

cocultured with HTR-8/SV40neo in the lower chamber. Concanamycin A (Sigma-Aldrich), an inhibitor of perforin, was used in cell culture to inhibit the cytotoxicity of perforin at working concentration of 50 nmol/L.

Detection of Apoptotic Cells

To assess apoptosis in spontaneous abortive tissue, a fluorescence terminal deoxynucleotidyl transferase dUTP nick-end labeling assay was performed according to the manufacturer's instructions (Apoptag *in situ* apoptosis detection kit; Oncor, Gaithersburg, MD). In brief, deparaffinized, dewaxed, and rehydrated sections were pretreated with 20 μg/ml proteinase K (Sigma-Aldrich) in 10 mmol/L Tris-HCl for 15 minutes, blocked with 10% normal goat serum, and then stained for terminal deoxynucleotidyl transferase dUTP nick-end labeling using a reaction mixture containing fluorescein-dUTP. Negative controls consisted of sections incubated without terminal deoxynucleotidyl transferase. Single immunohistochemical labeling using monoclonal antibodies against cleaved cytokeratin 18, M30 CytoDeath (Roche Diagnostics, Basel, Switzerland), after caspase-mediated cleavage, was performed according to the instructions provided with the Vectastain Elite ABC kit.(Vector Laboratories).

Flow Cytometry

One hundred microliters of a suspension of 1×10^6/ml of the decidual lymphocytes in PBS was first incubated with anti-CD56 PE (Becton Dickinson, San Jose, CA), permeabilized by incubating for 10 minutes with permeabilizing solution buffer (Becton Dickinson, dilute 10X solution 1:10 in deionized water), and then stained with biotin-labeled anti-granulysin mAb for 30 minutes at 4°C in the dark. Cells were washed and secondarily stained with fluorescein isothiocyanate-conjugated streptavidin (Becton Dickinson) for 30 minutes at 4°C in the dark. Cells were then washed, suspended in 500 μl of PBS, and immediately analyzed in a fluorescence-activated cell sorting (FACS) Callibur flow cytometer using the CellQuest program (Becton Dickinson). Other combination of antibodies was as follows: anti-CD3-PE (Becton Dickinson) and biotin-labeled anti-granulysin. We counted 15,000 cells in decidual lymphocytes.

For annexin V staining, HTR-8/SV40 cells transfected with GFP-control or GFP-granulysin vector were collected at 24 or 48 hours after transfection. The cells were then incubated with Alexa Fluor 594-conjugated annexin V (Molecular Probes, Eugene, OR) in an annexin V binding buffer (MBL Co. Nagoya, Japan) for 15 minutes at room temperature. Cells were washed, suspended in 500 μl of PBS, and immediately analyzed in a FACS Callibur flow cytometer using the CellQuest program. We counted the number of annexin-V positive cells in 20,000 GFP-positive cells.

Statistical Analysis

The Mann-Whitney *U* test was used for comparisons between two groups. Correlations were tested by single regression analyses. Values of $P < 0.05$ were considered statistically significant using Statview.

Results

Accumulation of Granulysin-Positive Cells in Decidua Basalis from Spontaneous Abortions

We first examined the expression of granule proteins such as granulysin, granzyme B, and perforin on spontaneous abortion tissues by immunohistochemistry. Immunohistochemistry for granulysin was seen in the decidua basalis, the region of implantation with the fertilized ovum, but was scant in the decidua parietalis, the region removed from the implantation site (Figure 2A, a and b). In spontaneous abortion, granulysin-positive lymphocytes were detected in not only the decidua basalis but also in the decidua parietalis (Figure 2A, c and d). Additionally, the majority of granulysin-positive cells were detected in cytotrophoblast and cell column, and a few in syncytiotrophoblast in spontaneous abortion. Confocal microscopic images also showed similar results in the granulysin expression on the decidua basalis in normal pregnancy and spontaneous abortion (Figure 2B, right panels). As shown in Figure 2C, the numbers of granulysin-positive cells in the spontaneous abortive tissues (decidua parietalis: 54.4 ± 14.4/HPF, median 53.5, range 37–70; decidua basalis: 216.9 ± 83.4/HPF, median 227.5, range 135–300) were significantly higher than those in normal pregnancy (decidua parietalis: 5.5 ± 3.7/HPF, median 5, range 2–10; decidua basalis: 37.8 ± 9.1/HPF, median 36.5, range 25–50) ($P = 0.002$ and $P = 0.0076$, respectively). On the other hand, there were no significant differences in the number of perforin-positive or granzyme B-positive cells either in the decidua parietalis or decidua basalis between spontaneous abortion and normal pregnancy (Figure 2C). These results showed that granulysin-positive cells, but not perforin or granzyme B-positive cells, accumulate at implantation sites in spontaneous abortion cases.

Granulysin-Expressing Decidual Lymphocytes Are CD 56^{bright} NK Cells

The accumulation of granulysin-positive cells in the decidua basalis was verified by immunohistochemistry, but it is still unclear which cells contained the granule protein in the decidua. On the assumption that decidual lymphocytes consisting mainly of NK cells and T cells contain granule proteins, we checked the expression of granulysin in decidual lymphocytes obtained from spontaneous abortion cases by immunohistochemistry or flow cytometry. Expression of granulysin was detected mainly in the cytoplasm of CD56-positive cells (Figure 3A). After exchanging additionally the first antibody for the second antibody to rule out the possibility of nonspecific binding, we obtained the same results (data not shown). We further examined the expression of granulysin in uterine CD56^{bright} NK cells or CD3$^+$ T cells of decidual lympho-