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.

International Journal of Gynecology and Obstetrics xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Gynecology and Obstetrics

journal homepage: www.elsevier.com/locate/ijgo

www.figo.org

BRIEF COMMUNICATION

Japanese single women have limited knowledge of age-related reproductive time limits

Mayumi Sugiura-Ogasawara *, Yasuhiko Ozaki, Saori Kaneko, Tamao Kitaori, Kyoko Kumagai

Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan

ARTICLE INFO

Article history:
Received 28 July 2009
Received in revised form 23 October 2009
Accepted 20 November 2009

Keywords:
Age
Knowledge of infertility
Reproductive time limit

In 2007, the total fertility rate in Japan decreased to 1.34, compared with 2.10 in the USA. This decline in fertility is partly due to increased age at marriage and delay in childbearing. Increased maternal age is associated with an increased risk of infertility, miscarriage, and poor pregnancy outcome [1]. A 1986 study found that the percentage of women who did not use contraception and remained childless increased steadily according to age at marriage (6% among 20–24-year-old women compared with 64% among 40–44-year-old women) [2]. The miscarriage rate rose from 14% among patients younger than 35 years of age to 40% among those older than 40 years of age [3]. The average childbearing age in Japan has increased over the past 3 decades as more women have postponed marriage to pursue higher education and careers.

Between June 2007 and March 2008, a 2-page anonymous survey was distributed at Nagoya City University, Nagoya Women's University, and a Women's Health Week in Nagoya (sponsored by the Japanese Society for Obstetrics and Gynecology). In total, 249 single women completed the 15-item survey, which contained questions addressing attitudes toward marriage, occupation, and childbirth, in addition to knowledge about infertility, miscarriage, and age-related reproductive time limits. The Research Ethics Committee at Nagoya City University Medical School approved the study.

The mean age of participants was 25.2 ± 6.8 years. In total, 95.5% of participants expressed a desire to marry in the future, with 96.8% wanting to continue work after marriage; however, 25.7% wanted to stop working after childbirth. Overall, 91.0% of women wanted to have children, and 85.5% thought that childbirth would be important in their life. The issues of marriage, career, and childbirth had been seriously considered by 46.2%, 67.9%, and 40.6% of single women, respectively. The mean age of women considering marriage and childbirth was higher than that of women not considering these events and of women who reported that they were influenced by parental values or common social practice (Table 1).

Nearly all women (98.8%) were familiar with the term "infertility," although only 44.2% and 17.3% chose accurate rates of infertility and miscarriage, respectively. Only 10.7% chose correct answers for all aspects of reproduction. Surprisingly, 36.4% estimated their own age limit for natural pregnancy to be between 45 and 60 years. Significantly more women considering marriage and childbirth chose correct responses about the rate of infertility and about reproduction. Women considering a career chose rates of infertility and miscarriage more accurately than did women not considering this option.

Regarding questions about the source of their knowledge of the term "infertility," 85.9% of women had learned the term from the media, compared with 20.7% who had learned it from school teachers. There was no difference in overall knowledge between women who obtained their information from school teachers and those who obtained it from other sources. Women considering marriage and those with accurate knowledge of infertility rates and causes tended to want children later. Participants with an accurate knowledge of infertility causes incorrectly chose a significantly older fertility time limit. Older participant age was significantly associated with knowledge of infertility rates; however, older participants wanted children later and believed reproductive time limits to be over 40 years of age.

In the present study, there were deficits in the participants' knowledge, despite the majority reportedly knowing the term "infertility." The terms "birth control," "contraception," "induced abortion," and "sexually transmitted infections" can be found in secondary-school health and physical education, biology, and domestic science textbooks, but "infertility" is seldom seen—indicating that there is no substantial public education in Japan about this condition. Women who reported that they had gained their knowledge of reproductive health from school teachers were unaware of accurate infertility rates. This raises concern that school teachers have limited knowledge on the subject, and indicates a need for infertility content to be added to the secondary-school curriculum.

The findings from the present study imply that, without knowing their reproductive limits, many Japanese women may lose their capacity for conception; thus, increased efforts are needed to educate

* Corresponding author. Mizuho-ku, Nagoya 4678601, Japan. Tel.: +81 52 853 8241; fax: +81 52 842 2269.
E-mail address: og.mym@med.nagoya-cu.ac.jp (M. Sugiura-Ogasawara).

0020-7292/$ – see front matter © 2009 Published by Elsevier Ireland Ltd. on behalf of International Federation of Gynecology and Obstetrics.
doi:10.1016/j.ijgo.2009.10.020

Please cite this article as: Sugiura-Ogasawara M, et al, Japanese single women have limited knowledge of age-related reproductive time limits, Int J Gynecol Obstet (2009), doi:10.1016/j.ijgo.2009.10.020

Table 1
Association between knowledge about reproductive health issues and personal aspirations[a].

	Age	Considering marriage	Considering career	Considering childbirth	School teachers listed as source of knowledge	Expected age at childbirth	Knowledge of reproductive time limit	Over 40 years of age
Age		<0.01	>0.05	<0.01				
		Yes 26.8±7.4	Yes 25.7±7.0	Yes 26.4±7.4				
		No 23.7±5.9	No 24.1±6.4	No 24.4±6.3				
Knowledge about infertility rate	0.01	<0.01	0.07	0.03	>0.05	0.08 (late)	>0.05	>0.05
Knowledge about male:female causes	>0.05	>0.05	0.05	>0.05	>0.05	0.07 (late)	0.01 (late)	>0.05
Knowledge about miscarriage rate	>0.05	>0.05	0.04	>0.05	>0.05	>0.05	>0.05	>0.05
Knowledge about all aspects of reproduction	0.07	0.04	>0.05	0.03	>0.05	0.09 (late)	>0.05	>0.05
Expected age at childbirth	<0.01 (late)	0.07 (late)	>0.05	>0.05	0.10 (early)			
Knowledge of reproductive time limit		>0.05	>0.05	>0.05	>0.05			
Over 40 years of age	0.06	>0.05	>0.05	>0.05	>0.05			
	Yes 25.4±6.9							
	No 24.8±6.5							

[a] Values are given as mean ± SD or *P* values.
[b] $P<0.05$ was considered statistically significant.

Japanese women about the influence of age on fertility. Societal pressures forcing women to choose between a career and children must change to reverse the very low fertility rate in Japan.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

This study was supported by the Japanese Ministry of Health, Labour, and Welfare.

References

[1] Gilbert WM, Nesbitt TS, Danielsen B. Childbearing beyond age 40: pregnancy outcome in 24,032 cases. Obstet Gynecol 1999;93(1):9–14.
[2] Menken J, Trussell J, Larsen U. Age and infertility. Science 1986;233(4771):1389–94.
[3] Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology, RESOLVE. 1999 Assisted Reproductive Technology Success Rates. Atlanta, GA: Centers for Disease Control and Prevention; 2001.

Please cite this article as: Sugiura-Ogasawara M, et al, Japanese single women have limited knowledge of age-related reproductive time limits, Int J Gynecol Obstet (2009), doi:10.1016/j.ijgo.2009.10.020

Journal of Reproductive Immunology 85 (2010) 186–192

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jreprimm

Antiphosphatidylethanolamine antibodies might not be an independent risk factor for further miscarriage in patients suffering recurrent pregnancy loss

Shintaro Obayashi[a], Yasuhiko Ozaki[a,*], Toshitaka Sugi[b], Tamao Kitaori[a], Kinue Katano[a], Sadao Suzuki[c], Mayumi Sugiura-Ogasawara[a]

[a] *Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Kawasumi 1, Mizuho-ku, Nagoya, Aichi 467-8601, Japan*
[b] *Sugi Women's Clinic, Laboratory for Recurrent Pregnancy Loss, Kanagawa, Japan*
[c] *Department of Public Health, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan*

ARTICLE INFO

Article history:
Received 17 December 2009
Received in revised form 19 February 2010
Accepted 3 March 2010

Keywords:
Antiphosphatidylethanolamine antibody
Recurrent pregnancy loss
β2-glycoprotein I-dependent anticardiolipin antibody
Lupus anticoagulant

ABSTRACT

The prevalence of antiphosphatidylethanolamine antibodies (aPEs) is higher in recurrent pregnancy loss patients than that in women with normal pregnancy. We conducted a cohort study to examine the predictive value of aPE for recurrent pregnancy loss and to determine its clinical significance. We examined plasma protein dependent (P+) and independent (P−) aPE IgG and IgM antibodies in 367 women with two or more unexplained consecutive pregnancy losses. We also examined conventional antiphospholipid antibodies (aPL) such as β2-glycoprotein I-dependent anticardiolipin antibodies (β2GPI-dependent aCL), lupus anticoagulant with reference to the dilute activated partial thromboplastin time (aPTT) and the diluted Russell's viper venom time (RVVT). Subsequent pregnancy outcome without medication was examined, and patients with and without aPE were compared. Totals of 37 (10.1%), 14 (3.8%), 23 (6.3%), 6 (1.6%), 9 (2.5%), 10 (2.7%) and 50 (13.6%) of the 367 patients were, respectively, positive for P+aPE IgG, P−aPE IgG, P+aPE IgM, P−aPE IgM, β2GPI-dependent aCL, lupus anticoagulant by RVVT and LA by aPTT. The patients with aPE differed from patients with β2GPI-dependent aCL or lupus anticoagulant by RVVT. No difference in live birth rate was apparent between positive and negative aPE patients with no medication. The areas under the curves for each ROC curve for the four aPEs were 0.535, 0.612, 0.546 and 0.533, respectively, so there was no significant variation in diagnostic capacity. We did not obtain any evidence that aPE elevation is an independent risk factor to predict further miscarriage in recurrent pregnancy loss patients.

1. Introduction

Established causes of recurrent pregnancy loss are abnormal chromosomes in either partner, particularly translocations, antiphospholipid antibodies (aPL) and uterine anomalies (Farquharson et al., 1984; Sugiura-Ogasawara et al., 2004, 2010). The antiphospholipid syndrome (APS) is the most important treatable etiology (Rai et al., 1997). The Sapporo criteria have been used to define APS since 1999 and preliminary classification criteria were revised more recently at a workshop in Sydney (Miyakis et al., 2006). With the new international criteria, patients can be diagnosed with APS when lupus anticoagulant and/or anticardiolipin antibodies (aCL) continue to be elevated for 12 weeks. Patients with persistent aPL should be treated with low dose aspirin and heparin combined therapy during pregnancy and about 70–80% can then experience a live birth (Rai et al., 1997).

* Corresponding author. Tel.: +81 52 853 8241; fax: +81 52 842 2269.
E-mail address: yozaki@med.nagoya-cu.ac.jp (Y. Ozaki).

0165-0378/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jri.2010.03.002

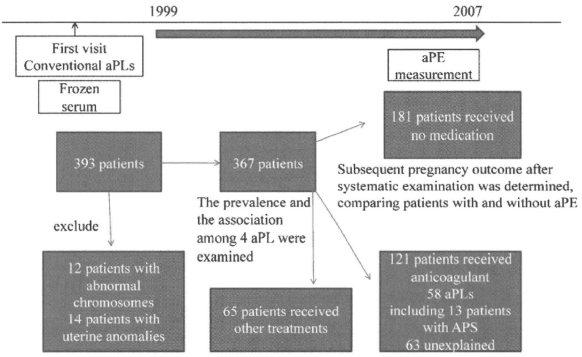


Fig. 1. Study profile.

Many kinds of aPL including antibodies against cardiolipin (CL), phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine (PE), have been measured by ELISA methods. β2-glycoprotein I (β2GPI) was found to be the true antigen of aCL in 1990 (Matsuura et al., 1990). Recently, aPL have been recognized to be autoantibodies to phospholipid-binding plasma proteins. The most common antigens are β2GPI and prothrombin (Roubey et al., 1992). β2GPI-dependent aCL, anti-β2GPI antibodies and lupus anticoagulant detected by the methods described in the International Thrombosis and Homeostasis Society are included in the International criteria for APS (Miyakis et al., 2006).

Sugi and McIntyre (1995) reported that certain antiphosphatidylethanolamine antibodies (aPEs) are not specific for PE per se but are directed to PE-binding plasma proteins, such as high molecular weight kininogen, low molecular weight kininogen, and proteins in complexes with kininogen, factor XI, or prekallikrein. The prevalence of aPE IgG and IgM were reported to be 20.1 and 12.2%, respectively, in patients suffering early pregnancy losses and significantly higher than in controls (Sugi et al., 1999). In contrast, rates for β2GPI-dependent aCL and lupus anticoagulant by dilute Russell's viper venom time (RVVT) were only 0.7 and 1.4%, respectively (Sugi et al., 1999).

However, to our knowledge, there are only limited data for any association between aPE and adverse pregnancy outcome in recurrent pregnancy loss cases (Gris et al., 2000). Thus, aPEs are not included in the international criteria for APS. We therefore here examined the predictive value of aPE and associations among β2GPI-dependent aCL and lupus anticoagulant for recurrent pregnancy loss to determine the clinical significance of aPE.

2. Materials and methods

2.1. Patients

Hysterosalpingography, chromosome analysis for both partners, determination of conventional aPLs, including both lupus anticoagulant by the 5 times diluted activated partial thromboplastin time (aPTT) method and the diluted RVVT method and β2GPI-dependent aCL, and blood tests for hyperthyroidism, diabetes mellitus and hyperprolactinemia were performed for all patients at the first visit of Nagoya City University Hospital. Serum for aPE measurement was taken at the same time when conventional aPL were measured and frozen at −70 °C. In total, we studied 367 women who had a history of two or more consecutive pregnancy losses. None of the patients had any readily identifiable causes of recurrent pregnancy loss, such as uterine or chromosomal abnormalities in either partner. None had received any medication before examination and there was no history of thrombosis. Their mean age was 31.9 ± 4.3 and the average number of previous early pregnancy losses was 2.7 ± 1.1. Twenty-two patients had a history of 26 events of intrauterine fetal death.

The patients' plasma protein dependent (P+) and independent (P−) aPE IgG and IgM were measured as aPE in 2007 using stored serum. aPE was measured once. The 367 pregnancies were recorded from August 1999 to December 2007 and subsequent pregnancy outcome was examined prospectively. A total of 58 patients were positive for at least one kind of conventional aPLs and 13 were diagnosed as APS, according to the Sapporo criteria and the Sydney revision. Sixty-three patients with unexplained causes were also treated with low dose aspirin and heparin therapy. Some 181 patients received no medication. Some 65 patients who received luteal support and a biological response modifier were excluded (Katano et al., 2000). The study profile is shown in Fig. 1.

Gestational age was calculated from basal body temperature charts. Ultrasonography was performed once or twice a week from 4 to 8 weeks' gestation. Dilation and curettage were carried out when miscarriages were diagnosed, and the karyotypes of aborted conceptuses were determined with the use of a standard G-banding technique. The present study was approved by the Research

Ethics Committee at Nagoya City University Medical School.

In the present study: (1) the prevalence and the association among 4 aPL were examined in 367 patients; (2) subsequent pregnancy outcome after systematic examination for pregnancy loss was determined in patients who received no medication or anticoagulants, comparing patients with and without aPE; (3) subsequent pregnancy outcome was examined excluding cases with abnormal karyotypes in aborted concepti; and (4) AUCs for ROC curves of aPE were calculated and receiver operating characteristic (ROC) analysis was carried out to ascertain whether aPE have predictive value for further miscarriage in 181 recurrent cases which received no medication.

2.2. Modified assays for the IgG and IgM isotypes against plasma protein binding phosphatidylethanolamine complex or phosphatidylethanolamine alone

Briefly, microtiter plates were coated with 30 μl of 50 μg/ml of PE (Aventi Polar Lipids, Birmingham, AL, USA), and each well was blocked for 1 h with 10% bovine serum albumin. To detect phospholipid-binding plasma protein dependent and independent reactivity, 50 μl aliquots of patient plasma diluted 1:100 containing either 10% adult bovine plasma or 1% bovine serum albumin were incubated for 1 h. Antibodies were detected with alkaline phosphatase labeled anti-human IgG or IgM antibodies. Nonspecific binding control wells were processed in parallel and the background values were subtracted (Sugi et al., 1999).

Cut-off levels were set at mean ± 2SD, established using sera from 122 healthy volunteers. Therefore 0.32, 0.45, 0.44 and 1.0 were considered positive for P+ aPE IgG, P − aPE IgG, P + aPE IgM and P − aPE IgM, respectively.

2.3. Assay for the lupus anticoagulant by the diluted aPTT method

Brain cephalin (Automated aPTT, Organon Teknica, Durham, NC) was employed as a phospholipid reagent for the determination of aPTT, diluted 5 times in veronal saline (Ogasawara et al., 1996a).

A: Fifty μl of non-pregnant control woman plasma, 50 μl of standard plasma, and 100 μl of diluted cephalin were mixed and incubated for exactly 3 min at 37 °C. B: At the same time 100 μl of standard plasma alone and 100 μl of diluted cephalin were mixed and incubated for 3 min at 37 °C. One hundred microlitres of 25 mM $CaCl_2$ was added and clotting time was measured with an Option 4 bioMeriux calculator, France.

Clotting times for A–B with control plasma samples from 104 healthy non-pregnant women were first examined. The mean and standard deviation values were 2.57 and 1.60 s, respectively. Lupus anticoagulant was considered positive when prolonged clotting times (>mean ± 3SD, 7.37 s) failed to correct when samples were mixed 1:1 with standard plasma.

2.4. Assay for the lupus anticoagulant with reference to the diluted Russell's viper venom time

To determine *T*1, 200 μl of healthy non-pregnant control woman plasma and 200 μl of diluted Russell viper venom and phospholipid reagents containing 25 mM $CaCl_2$ (Gradipore Ltd., Pyrmont, Australia) were mixed and clotting time was measured with an Option 4 bioMeriux calculator, France. To determine *T*2, 200 μl of the same non-pregnant control plasma and 200 μl of diluted Russell's viper venom and phospholipid-rich reagents containing 25 mM $CaCl_2$ were mixed and clotting time was measured. The mean and standard deviation values were 0.9 and 0.1 s, respectively. Lupus anticoagulant was considered positive when *T*1/*T*2 was over 1.3.

2.5. Modified assays for the IgG isotypes of β2GPI-dependent and -independent aCLs

Briefly, cardiolipin in ethanol (2.5 μg/50 μl/well) was coated onto the surfaces of polystyrene microtiter plates by evaporation under nitrogen. For the detection of β2GPI-dependent aCL, duplicate wells were incubated with 50 μl of HEPES–BSA, containing purified human β2GPI (30 μg/ml; Yamasa Corp., Choshi, Japan), for 10 min at room temperature. For the determination of β2GPI-independent aCL, duplicate wells were incubated with 50 μl of HEPES–BSA in the same manner. Fifty microlitres of test sera, diluted 1:202 in HEPES–BSA, were then introduced into the duplicate wells and incubations were performed for 30 min at room temperature. After washing with PBS–Tween, wells were exposed to 100 μl of horseradish peroxidase-labeled murine monoclonal IgG against human IgG (G-02; Yamasa Corp.) for 30 min at room temperature. After washing, a 100 μl aliquot of 0.3 mM tetramethylbendizine solution containing 0.003% of H_2O_2 was added to each well. The reaction was terminated by adding 100 μl of 2N H_2SO_4, and the optical density was measured at 450 nm. Antibody titers (units/ml) of aCL were calculated from a standard curve, obtained by running six calibration standards (Yamasa Corp., 1.3–125 units/ml) for each plate.

Test results for β2GPI-dependent and -independent aCL were considered positive when the antibody level was above the 99% confidence interval for 283 normal non-pregnant control sera. This was more than 1.9 units/ml for β2GPI-dependent and more than 6.3 units/ml for β2GPI-independent aCL. In addition, in order to avoid false positives due to nonspecific binding, a β2GPI-dependent assay had to show a higher value than the β2GPI-independent assay performed in parallel, to be considered positive (Matsuura et al., 1994; Katano et al., 1996).

2.6. Statistical analysis

Receiver operating characteristics (ROC) curves for each PE were drawn for all cut-off points. In order to examine the diagnostic values for each PE, areas under the curves (AUCs) for ROC curves were calculated. The analysis was carried out with the PROC LOGISTIC procedure in SAS sys-

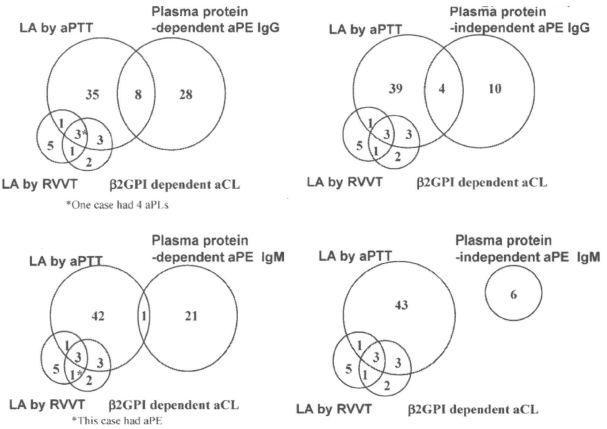


Fig. 2. Overlapping associations among the 4 aPLs for the 367 patients with a history of recurrent pregnancy loss.

tem version 9.1 (SAS Institute Inc., NC, USA) with $P < 0.05$ considered to be statistically significant.

3. Results

Totals of 37 (10.1%), 14 (3.8%), 23 (6.3%), 6 (1.6%), 9 (2.5%), 10 (2.7%) and 50 (13.6%) of the 367 patients were, respectively, positive for P+aPE IgG, P−aPE IgG, P+aPE IgM, P−aPE IgM, β2GPI-dependent aCL, lupus anticoagulant by RVVT and lupus anticoagulant by aPTT.

The relations among aPE and conventional aPL are shown in Fig. 2. Patients with P+aPE IgG were separated from those with β2GPI-dependent aCL and lupus anticoagulant by RVVT. Only one case was positive for all tests. Eight of 37 patients had both P+aPE IgG and lupus anticoagulant by aPTT. On the other hand, only one patient had P+aPE IgM and LA. Six patients with P−aPE IgM were completely separated from conventional aPL.

Eighty-eight of 367 (24.0%) patients miscarried again. Characteristics and subsequent pregnancy outcome for all 302 patients who received no medication or anticoagulant are given in Table 1. With regard to the no medication group, 10 of 14 patients (71.4%) positive for P+aPE IgG gave birth to living babies, while 127 of 167 patients (76.0%) negative for P+aPE IgG had successful pregnancies (difference not significant). A total of 4 of 7 patients (57.1%) positive for P−aPE IgG gave birth to living babies, while 133 of 174 patients (76.4%) negative for P−aPE IgG had successful pregnancies (difference not significant).

Fifty-five karyotypes of miscarried concepti could be analyzed and 31 (56.4%) were found to be abnormal. After excluding miscarriage cases caused by an abnormal embryonal karyotype, the success rate (83.3%) of patients positive did not differ from that (83.6%) of patients negative for P+aPE IgG.

AUCs for each ROC curve, as shown in Fig. 2, for P+aPE IgG, P−aPE IgG, P+aPE IgM and P−aPE IgM was 0.535, 0.612, 0.546 and 0.533, respectively. Each AUC was close to 0.5 so that there was no variation in diagnostic capacity of the test. These results thus did not suggest any significant predictive value of 4 aPE for further miscarriage (Fig. 3).

4. Discussion

In the present study, the population with aPE was found to differ substantially from those with β2GPI-dependent aCL and lupus anticoagulant by RVVT. Only 8 patients had both P+PE IgG and lupus anticoagulant by aPTT. It is well-known that purified IgG from patients with lupus anticoagulant has lupus anticoagulant activity, thus patients with lupus anticoagulant by aPTT had not aPE IgM but aPE IgG in the present study. aPTT influences the intrinsic pathway including the contact phase cascade and, in contrast, RVVT inhibits coagulation factor X directly. Lupus

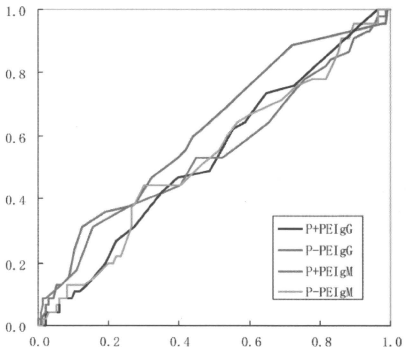


Fig. 3. ROC curves for anti-PE antibodies.

Table 1
Characteristics and pregnancy outcome of patients with antiphosphatidylethanolamine antibodies.

	No medication		Anticoagulant	
	Positive	Negative	Positive	Negative
P + aPE IgG	$n = 14$	$n = 167$	$n = 12$	$n = 109$
Mean age	31.3	31.6	34.9	32.6
Mean no. of previous losses	2.2	2.3	3.0	3.1
Failure	4 (2)	40 (25)	4 (2)	26 (17)[a]
Success	10	127	8	83
Success rate (%)	71.4 (83.3)	76.0 (83.6)	66.7 (80.0)	76.1 (83.0)[b]
P − aPE IgG	$n = 7$	$n = 174$	$n = 5$	$n = 116$
Mean age	33.0	31.5	32.0	37.8
Mean no. of previous losses	2.0	2.3	3.0	3.1
Failure	3 (1)	41 (26)	2 (2)	28 (17)
Success	4	133	3	88
Success rate (%)	57.1 (80.0)	76.4 (83.6)	60.0 (60.0)	75.9 (83.8)
P + aPE IgM	$n = 6$	$n = 175$	$n = 11$	$n = 110$
Mean age	31.7	31.5	32.2	32.5
Mean no. of previous losses	2.0	2.3	3.0	3.2
Failure	4 (3)	40 (24)	2 (1)	28 (18)
Success	2	135	9	82
Success rate (%)	33.3 (40.0)	77.1 (84.9)	81.8 (90.0)	74.5 (82.0)
P − aPE IgM	$n = 2$	$n = 179$	$n = 2$	$n = 119$
Mean age	30.5	31.4	29.5	32.7
Mean no. of previous losses	3.0	2.0	2.5	3.1
Failure	0	44 (27)	0	30 (19)
Success	2	135	2	89
Success rate (%)	100 (100)	75.4 (83.3)	100	74.8 (82.4)

[a] Miscarriages caused by an abnormal embryonal karyotype were excluded.
[b] Success rate when miscarriages caused by an abnormal embryonal karyotype were excluded.

anticoagulant comprises heterogeneous antibodies against phospholipid-binding prothrombin, factor X and/or β2GPI (Bever et al., 1991; Brandt, 1991; Roubey et al., 1992). Thus, lupus anticoagulant by aPTT included β2GPI-dependent aCL and lupus anticoagulant by RVVT. aPE can recognize kininogen–PE complexes (Sugi and McIntyre, 1995). Lupus anticoagulant acting by aPTT but not by RVVT might comprise IgG against kininogen–PE complexes.

Antigenic targets include β2GPI, prothrombin, high and low molecular weight kininogen, annexin V, protein C and protein S (Roubey et al., 1992; Roubey, 1994). In addition to approaches for conventional aPLs, new ELISA methods for aPE, anti-prothrombin and anti-annexin V antibodies are now available (Bever et al., 1991; Sugi and McIntyre, 1995; Matsubayashi et al., 2001). We have shown that β2GPI-dependent aCL is a strong predictor of intrauterine fetal death, intrauterine growth restriction and pregnancy-induced hypertension, although the frequency is low (Katano et al., 1996). These conventional aPLs are included in the international criteria for APS. However, the prevalence of β2GPI-dependent aCL and lupus anticoagulant by dRVVT are relatively low (2.5 and 2.7% in the present study) and Sugi et al. (1999) have concluded that aPEs are more strongly associated with recurrent pregnancy loss because the prevalence of PE IgG and IgM were found to be much higher (20.1 and 12.2%).

However, a high prevalence in a particular test does not necessarily imply clinical significance. With regard to antinuclear antibodies (ANA), the frequency is significantly higher than in controls, but no effects on the live birth rate were found in one study (Ogasawara et al., 1996b).

Moreover, it is unlikely that all these molecules are targeted at the same time; the whole situation rather reflects the extensive immunologic alterations that characterize the pregnant status. The exact role of even β2GPI itself in pregnancy loss remains unknown because knockout mice are fertile (Miyakis et al., 2004).

To our knowledge there have hitherto been only a few reports of the predictive value of aPE for adverse pregnancy outcome of recurrent aborters. Gris et al. (2000) described aPE IgM but not IgG to have predictive value for subsequent fetal loss from the 8th week up to and including the 22nd week of gestation, in spite of low dose aspirin treatment. Recently, Yamada et al. (2009) measured aPLs including aPE IgG during the first trimester in a consecutive series of 1155 pregnant women and found that aPE IgG was associated with developing pregnancy-induced hypertension (8.3, 2.4–29) and premature delivery (12.7, 3.1–50). However, they could not examine the association between aPE IgG and early miscarriage because the peripheral blood was obtained at 8–14 weeks' gestation.

Another issue that relates to aPE and most of the aPL is the lack of standardization and of uniform requirements for performance and interpretation of the tests. From comparing the detection methods for aPE in the present study with those in Gris's study, there are some important differences (for example, regarding overnight incubation and methanol dilutions of aPE plates, extraction of standards for OD estimation). Similar limitations and particularities exist for the measurements of most of the aPLs. Using different detection methodologies can cause different prevalence values, even though the same aPL are purportedly mea-

sured. Another limitation is that we did not measure persistent aPE though International criteria recommend two times measurement.

Our present study, the first to examine the predictive value of aPE in recurrent pregnancy loss patients, failed to prove any significant link with an adverse outcome in untreated patients. We could not examine subsequent pregnancy loss of the 8 cases with both P+aPE IgG and lupus anticoagulant by aPTT without medication and we have proved that the live birth rate in patients with a history of recurrent miscarriage who had lupus anticoagulant by aPTT was improved from 46.2 to 80.4% by anticoagulant therapy (Ogasawara et al., 2001). It is unclear whether P+aPE IgG might have a predictive value if the 8 patients with no medication were followed up. This is not a case–control study but a cohort study. Patients with APS, occasional aPL and unexplained causes were treated with anticoagulant. Thus, it is difficult to make a simple direct comparison between the two groups with and without anticoagulant. Further study is needed excluding the influence of lupus anticoagulant by aPTT because lupus anticoagulant by aPTT cannot be examined commercially, although tests for aPE, β2GPI-dependent aCL and lupus anticoagulant by dRVVT are commercially obtainable and widely applied in Japan.

The prevalence of aPE IgG and aPE IgM were earlier reported to be 20.1 and 12.2% (Sugi et al., 1999). The prevalence of aPE IgG included P+aPE IgG and P−aPE IgG and the prevalence of P+aPE IgG and P+aPE IgM were 11.5 and 5.8%, respectively. These were 10.1 and 6.3% in the present study, where we performed ELISA using not purified kininogen but rather bovine plasma obtainable commercially. We should focus on kininogen dependence because a previous study showed a link with kininogen–PE IgG (Sugi and McIntyre, 1995; Sugi et al., 1999). The contact activation system consists of coagulation factor XII, kallikrein and high molecular weight kininogen. Factor XII can be activated by contact with negatively charged surfaces and factor XIIa then converts prekallikrein to kallikrein. Kallikrein digests kininogen to liberate the vasoactive, proinflammatory mediator, bradykinin. Factor XIIa also stimulates factor XI, which activates the intrinsic coagulation pathway. Decreased factor XII activity is also associated with recurrent miscarriage (Ogasawara et al., 2001). The presence of low molecular weight kininogen and high molecular weight kininogen in the porcine uterus and endometrial gene expression of plasma kallikrein and factor XII provide evidence that the kallikrein–kininogen–kinin system is biologically active during establishment of pregnancy in the pig (Vonnahme et al., 2004). Alternatively, kininogen-dependent PE IgG might thus be associated with early spontaneous abortion by the inhibition of bradykinin-induced relaxation in uteral arteries (Kenny et al., 2002).

In conclusion, we did not obtain any evidence that aPE predicts further miscarriage in recurrent pregnancy loss patients. However, a further study is needed for confirmation because the sample size of the population with no medication was too small. Improvements are also required for the commercially obtained assay of aPE in common use in Japan and an investigation is clearly warranted to exclude influence of lupus anticoagulant by aPTT because it identifies a different population from those with conventional aPL.

Acknowledgment

The authors thank Hiroko Johnokuchi (Department of Food Science and Nutrition, Faculty of Human Life and Environmental Sciences, Nagoya Women's University) for her technical support.

References

Bever, E.M., Galli, M., Barbui, T., Comfurius, P., Zwaal, R.F.A., 1991. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb. Haemost. 66, 629–632.

Brandt, J.T., 1991. Assays for phospholipid-dependent formation of thrombin and Xa: a potential method for quantifying lupus anticoagulant activity. Thromb. Haemost. 66, 453–458.

Farquharson, R.G., Pearson, J.F., John, L., 1984. Lupus anticoagulant and pregnancy management. Lancet 28, 228–229.

Gris, J.C., Quéré, I., Sanmarco, M., Boutiere, B., Mercier, E., Amiral, J., Hubert, A.M., Ripart-Neveu, S., Hoffet, M., Tailland, M.L., Rousseau, O., Monpeyroux, F., Dauzat, M., Sampol, J., Daures, J.P., Berlan, J., Marès, P., 2000. Antiphospholipid and antiprotein syndromes in non-thrombotic, non-autoimmune women with unexplained recurrent primary early foetal loss. The Nimes Obstetricians and Haematologists Study—NOHA. Thromb. Haemost. 84, 228–236.

Katano, K., Aoki, A., Sasa, H., Ogasawara, M., Matsuura, E., Yagami, Y., 1996. Beta 2-glycoprotein I-dependent anticardiolipin antibodies as a predictor of adverse pregnancy outcomes in healthy pregnant women. Hum. Reprod. 11, 509–512.

Katano, K., Aoki, K., Ogasawara, M.S., Suzumori, K., 2000. Adverse influence of numbers of previous miscarriages on results of paternal lymphocyte immunization in patients with recurrent spontaneous abortions. Am. J. Reprod. Immunol. 44, 289–292.

Kenny, L.C., Baker, P.N., Kendall, D.A., Randall, M.D., Dunn, W.R., 2002. Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia. Clin. Sci. 103, 67–73.

Matsubayashi, H., Arai, T., Izumi, S., Sugi, T., McIntyre, J.A., Makino, T., 2001. Anti-annexin V antibodies in patients with early pregnancy loss or implantation failures. Fertil. Steril. 76, 694–699.

Matsuura, E., Igarashi, Y., Fujimoto, M., Ichikawa, I., Koike, T., 1990. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. Lancet 21, 177–178.

Matsuura, E., Igarashi, Y., Yasuda, T., Triplett, D.A., Koike, T., 1994. Anticardiolipin antibodies recognize β2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. J. Exp. Med. 179, 457–462.

Miyakis, S., Robertson, S.A., Krillis, S.A., 2004. Beta-2 glycoprotein I and its role in antiphospholipid syndrome-lessons from knockout mice. Clin. Immunol. 112, 136–143.

Miyakis, S., Lockshin, M.D., Atsumi, T., Branch, D.W., Brey, R.L., Cervera, R., Derksen, R.H.W.M., De Groot, P.G., Koike, T., Meroni, P.L., Reber, G., Shoenfeld, Y., Tincani, A., Vlachoyiannopoulos, P.G., Krilis, S.A., 2006. International consensus statement of an update of the classification criteria for definite antiphospholipid syndrome (APS). J. Thromb. Haemost. 4, 295–306.

Ogasawara, M., Aoki, K., Matsuura, E., Sasa, H., Yagami, Y., 1996a. Anti β2glycoprotein I antibodies and lupus anticoagulant in patients with recurrent pregnancy loss: prevalence and clinical significance. Lupus 5, 587–592.

Ogasawara, M., Aoki, K., Kajiura, S., Yagami, Y., 1996b. Are antinuclear antibodies predictive of recurrent miscarriage? Lancet 347, 1183–1184.

Ogasawara, M., Aoki, K., Katano, K., Ozaki, Y., Suzumori, K., 2001. Factor XII but not protein C, protein S, antithrombin III or factor XIII as a predictor of recurrent miscarriage. Fertil. Steril. 75, 916–919.

Rai, R., Cohen, H., Dave, M., Regan, L., 1997. Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). BMJ 314, 253–257.

Roubey, R.A.S., Pratt, C.W., Buyon, J.P., Winfield, J.B., 1992. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon β2glycoprotein I. J. Clin. Invest. 90, 1100–1104.

Roubey, R.A.S., 1994. Autoantibodies to phospholipid-binding plasma proteins: a new view of lupus anticoagulant and other "antiphospholipid" autoantibodies. Blood 84, 2854–2867.
Sugi, T., McIntyre, J.A., 1995. Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen–PE complex. Blood 86, 3083–3089.
Sugi, T., Katsunuma, J., Izumi, S., McIntyre, J.A., Makino, T., 1999. Prevalence and heterogeneity of antiphosphatidylethanolamine antibodies in patients with recurrent early pregnancy losses. Fertil. Steril. 71, 1060–1065.
Sugiura-Ogasawara, M., Ozaki, Y., Sato, T., Suzumori, N., Suzumori, K., 2004. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocation. Fertil. Steril. 81, 367–373.
Sugiura-Ogasawara, M., Ozaki, Y., Kitaori, T., Kumagai, K., Suzuki, S., 2010. Midline uterine defect size correlated with miscarriage of euploid embryos in recurrent cases. Fertil. Steril. 93, 1983–1988.
Yamada, H., Atsumi, T., Kobashi, G., Ota, C., Kato, E.H., Tsuruga, N., Ohta, K., Yasuda, S., Koike, T., Minakami, H., 2009. Antiphospholipid antibodies increase the risk of pregnancy induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. 79, 188–195.
Vonnahme, K.A., Fernando, S.C., Ross, J.W., Ashworth, M.D., DeSilva, U., Malayer, J.R., Geisert, R.D., 2004. Porcine endometrial expression of kininogen, factor XII, and plasma kallikrein in cyclic and pregnant gilts. Biol. Reprod. 70, 132–138.

OPEN ACCESS Freely available online

PLoS one

Stem Cell-Like Properties of the Endometrial Side Population: Implication in Endometrial Regeneration

Hirotaka Masuda[1,2], Yumi Matsuzaki[1]*, Emi Hiratsu[1], Masanori Ono[2], Takashi Nagashima[2], Takashi Kajitani[2], Toru Arase[2], Hideyuki Oda[2], Hiroshi Uchida[2], Hironori Asada[2], Mamoru Ito[3], Yasunori Yoshimura[2], Tetsuo Maruyama[2]*, Hideyuki Okano[1]

1 Department of Physiology, Keio University School of Medicine, Tokyo, Japan, **2** Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan, **3** Central Institute for Experimental Animals, Kanagawa, Japan

Abstract

Background: The human endometrium undergoes cyclical regeneration throughout a woman's reproductive life. Ectopic implantation of endometrial cells through retrograde menstruation gives rise to endometriotic lesions which affect approximately 10% of reproductive-aged women. The high regenerative capacity of the human endometrium at eutopic and ectopic sites suggests the existence of stem/progenitor cells and a unique angiogenic system. The objective of this study was to isolate and characterize putative endometrial stem/progenitor cells and to address how they might be involved in the physiology of endometrium.

Methodology/Principal Findings: We found that approximately 2% of the total cells obtained from human endometrium displayed a side population (SP) phenotype, as determined by flow cytometric analysis of Hoechst-stained cells. The endometrial SP (ESP) cells exhibited preferential expression of several endothelial cell markers compared to endometrial main population (EMP) cells. A medium specific for endothelial cell culture enabled ESP cells to proliferate and differentiate into various types of endometrial cells, including glandular epithelial, stromal and endothelial cells *in vitro*, whereas in the same medium, EMP cells differentiated only into stromal cells. Furthermore, ESP cells, but not EMP cells, reconstituted organized endometrial tissue with well-delineated glandular structures when transplanted under the kidney capsule of severely immunodeficient mice. Notably, ESP cells generated endothelial cells that migrated into the mouse kidney parenchyma and formed mature blood vessels. This potential for *in vivo* angiogenesis and endometrial cell regeneration was more prominent in the ESP fraction than in the EMP fraction, as the latter mainly gave rise to stromal cells *in vivo*.

Conclusions/Significance: These results indicate that putative endometrial stem cells are highly enriched in the ESP cells. These unique characteristics suggest that ESP cells might drive physiological endometrial regeneration and be involved in the pathogenesis of endometriosis.

Citation: Masuda H, Matsuzaki Y, Hiratsu E, Ono M, Nagashima T, et al. (2010) Stem Cell-Like Properties of the Endometrial Side Population: Implication in Endometrial Regeneration. PLoS ONE 5(4): e10387. doi:10.1371/journal.pone.0010387

Editor: Manuel Tena-Sempere, University of Córdoba, Spain

Received October 27, 2009; **Accepted** March 25, 2010; **Published** April 28, 2010

Funding: This study was supported, in part, by the Ministry of Education, Science, and Culture of Japan (to T.M., Y.Y., and H.M.), by a National Grant-in-Aid for the Establishment of a High-Tech Research Center in a Private University (to T.M.) and by a grant-in-aid from the Global Centers of Excellence program of the Ministry of Education, Science, and Culture of Japan to Keio University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: penguin@sc.itc.keio.ac.jp (YM); tetsuo@sc.itc.keio.ac.jp (TM)

Introduction

Human endometrium, which lines the uterine cavity, exhibits unique properties of cyclical regeneration and tissue breakdown under the influence of estrogen and progesterone throughout the course of a woman's reproductive life. Retrograde shedding and ectopic implantation of menstrual endometrial cells and tissue fragments give rise to endometriotic lesions outside of the uterus. Endometrial cells prepared from the human endometrium are also capable of reconstituting functional endometrium in xenograft models of endometriosis [1]. When single cell suspensions of endometrial cells are transplanted under the kidney capsule of severely immunodeficient NOD/SCID/γ_c^{null} (NOG) mice [1], the reconstructed ectopic endometrial tissues show menstrual cycle-related morphological and functional changes repeatedly in response to treatment with estrogen and progesterone [1]. These unique properties reflect the remarkable capacity of human endometrial cells for regeneration at eutopic and ectopic locations, and suggest the existence of stem/progenitor cells as well as an angiogenic system in the human endometrium. Indeed, it has been postulated that the endometrium contains a pool of multipotent stem cells within the deep *basalis* layer, capable of cyclically producing progenitor cells that further differentiate into each endometrial cell component [2,3].

Several groups have identified a number of endometrial cell subpopulations as candidate endometrial stem/progenitor cells. These include clonogenic endometrial cells [4], endometrial SP cells which possess a Hoechst 33342 low-fluorescence profile [5,6], $CD146^+PDGFR\beta^+$ stromal cells [7], and $CD29^+CD73^+CD90^+$ stromal cells [8]. The phenotypic and functional stem cell-like

properties, however, have only been characterized *in vitro*. No studies have yet explored the *in vivo* regenerative capacity of these putative endometrial stem/progenitor cells. Candidate tissue-specific stem cells have been identified in several tissues based on the SP phenotype. This characteristic is due to the unique ability of the primitive cells to pump out the DNA binding dye Hoechst 33342 via the ATP-binding cassette transporter G2 (ABCG2) [9–12]. Primitive hematopoietic precursors from bone marrow were the first SP cells identified with this technique [13]. We recently demonstrated that SP cells isolated from the human uterine myometrium regenerate human myometrial tissues *in vivo* when xenotransplanted into the uteri of NOG mice [14].

In the present study, we adapted our *in vivo* regeneration assay and SP isolation procedure to characterize the properties of human endometrial SP (ESP) cells. These cells were able to differentiate into endometrium-like tissue and a variety of endometrial cell components when xenotransplanted into NOG mice. This is the first *in vivo* evidence in support of the existence of stem/progenitor cells in the ESP.

Results

Isolation of ESP and endometrial main population (EMP) cells from human cycling endometrium

We first dissociated human endometria mechanically and enzymatically and purified epithelial-enriched and stromal-enriched fractions as previously described [1]. Procedures for preparing these two fractions are summarized schematically in Figure 1A. Both fractions were then stained with Hoechst dye and subjected to flow cytometric analysis and cell sorting.

We found that each preparation contained a small subset of cells in the SP fraction (Figure 1B). SP cells constituted $2.741 \pm 0.443\%$ (mean ± SEM, n = 43) of the viable cells in the epithelial-enriched fraction, whereas SP cells represented $3.091 \pm 0.439\%$ (mean ± SEM, n = 43) of the stromal-enriched preparation. The appearance of the SP populations was blocked by 50 μM reserpine (Figure 1B, insets), a general characteristic of SP cells [15]. Since it was unclear which SP fraction contained the endometrial stem/progenitor cells, we mixed the epithelial and stromal SP cells or isolated SP cells from the mixture of the two fractions. We designated the SP and main population (MP) cells derived from both fractions as ESP and EMP, respectively. Endometrial replicative population was designated as ERP (Figure 1B). The ESP and EMP cells were then used for further experiments. The ESP cells represented $2.832 \pm 0.326\%$ (mean ± SEM, n = 52) of the total living endometrial cells (Figure 1B).

We next examined whether the proportion of ESP cells varied across the menstrual cycle. As shown in Figure 1C, the proportion of ESP cells to the EMP + ERP fraction was the highest at the early proliferative phase, decreasing gradually until its nadir in the late secretory phase. This may reflect an increase in the number of EMP cells from menstruation towards the late secretory phase.

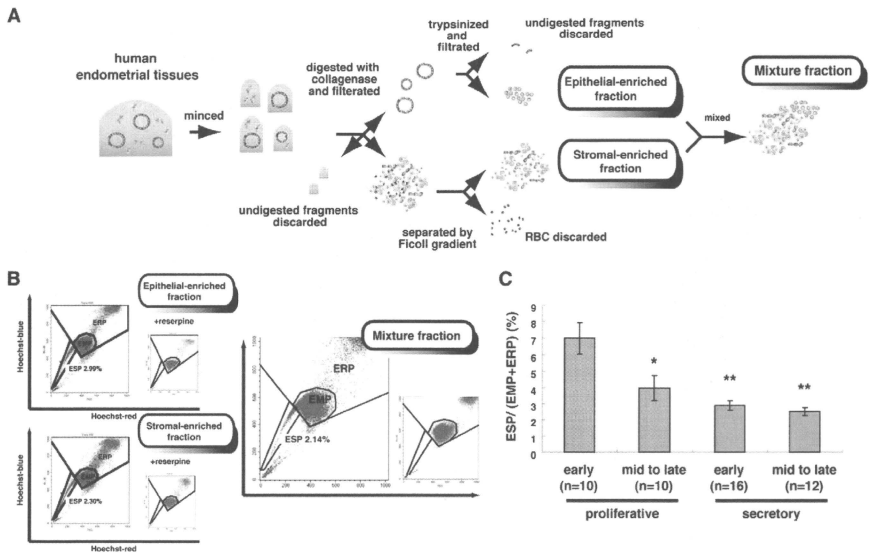


Figure 1. Isolation of ESP and EMP cells. (**A**) Summary of procedures for the preparation of epithelial-enriched and stromal-enriched fractions and the mixture of both fractions from human cycling endometria. (**B**) Flow cytometric distribution of ESP cells, EMP cells, and the endometrial replicative population (ERP) in each of the three fractions stained with Hoechst 33342 indicated in Figure S1. Addition of 50 μM reserpine resulted in the disappearance of the ESP fraction (inset in each panel). (**C**) Proportion of ESP in the whole fraction dissociated from human endometria at different phases of the menstrual cycle. * $P<0.01$ and ** $P<0.00005$, versus early proliferative phase. Each bar indicates the mean ± SEM. n = 48.
doi:10.1371/journal.pone.0010387.g001

In vivo reconstitution activity of ESP and EMP cells

To investigate the stem cell-like regenerative capabilities of ESP cells *in vivo*, we transplanted ESP cells under the kidney capsule of ovariectomized NOG mice, after which they were treated with a 17β-estradiol (E_2) pellet for eight to ten weeks. As was seen in the endometrial regeneration model [1], ESP cells, but not EMP cells, generated a cystic mass (Figure 2A) with delineated glandular and stromal structures at the site of transplantation (Figure 2B). However, the reconstitution efficiency was low in that the reconstitution of epithelial and stromal endometrial-like tissues was observed in 2 out of 24 xenotransplanted mice (Figure 2C).

No macroscopically identifiable masses were generated in the remaining 22 mice transplanted with ESP cells; however, histological and microscopic analyses of the transplantation sites revealed the existence of three distinctive tissue subtypes within this area. Immunofluorescence studies revealed that the newly formed tissue, but not the mouse kidney, stained with human vimentin (Vm) antibody (clone V9) (Figure 2C). As V9 can recognize only human Vm (hVm), the new tissue was clearly of human origin. These we designated as the "vascular endothelial type", "migrating endothelial type" and "stromal type", based on the dominant type of human-originated structures or cell components present in the transplanted site (Figure 2C). In the "vascular endothelial type", vessel-like structures consisting of hVm^+ cells were dominant, together with some hVm^+ cells migrating into the mouse kidney parenchyma (Figure 2C). We confirmed that most of the migratory hVm^+ cells were positive for CD31 (Figure S1A). Furthermore, ESP-derived $CD31^+$ endothelium was surrounded by a layer of ESP-derived smooth muscle cells positive for α-smooth muscle actin (αSMA) (Figure 2D). In the "migrating endothelial type", migratory cells positive for both hVm and CD31 were dominant in the interspaces of the murine kidney parenchyma (Figure S1A). This type was frequently accompanied by stromal cell layers but not by vessel-like structures. These results collectively suggest that ESP cells have the potential to differentiate into not only small capillary vessels but also mature arteries and veins. The "stromal type" showed layers of fibroblastic hVm^+ cells, which were also positive for an endometrial stromal cell marker, CD13 (Figure S1B). Notably, the heterogeneity of the regenerated tissue or cell components was more evident in kidneys transplanted with ESP cells than in those transplanted with EMP cells. This indicates that ESP cells may have a broader differentiative capability than cells in the EMP fraction (Figure 2C), as the latter mainly gave rise to stromal cells *in vivo*.

The glandular structure of the well-organized tissue was positive for cytokeratin (CK), an epithelial marker (Figure 2E). The regenerated tissue also contained human CD31-positive endothelial cells (Figure 2F). It is well known that the endometrial progesterone receptor (PR) is upregulated by E_2 stimulation. In agreement, PR was expressed prominently in the E_2-exposed endometrial tissue (Figure 2G). These results collectively suggested that ESP cells had the potential to regenerate functional endometrium; however, the efficiency of this process was low

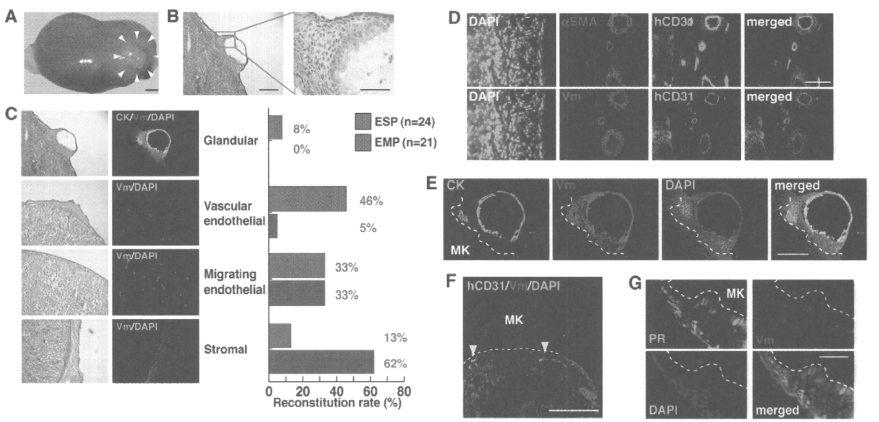


Figure 2. Recreation of the human endometrium and its components from ESP cells in immunodeficient mice. (**A**) The macroscopic appearance of an ESP-initiated lesion (surrounded by white arrowheads) in the kidney of a NOD/SCID/γ_c^{null} (NOG) mouse treated with E_2 pellets for 10 weeks. Bar, 1 mm. (**B**) H&E-stained section of the same lesion indicated in (**A**). A small box marks a region shown at a higher magnification in the adjacent panel as indicated. Bars, 500 μm (left) and 100 μm (right). (**C**) H&E-stained and immunofluorescence images of ESP- or EMP-initiated lesions co-stained with DAPI and antibodies against CK and Vm where various endometrial cell components were formed. The right table shows the number and frequency of the ESP- or EMP- initiated mice which predominantly possessed human endometrium with glandular structures, vessel-like structures consisting of endothelial cells, migrating endothelial cells, or stromal cell components. Bars, 500 μm. (**D**) Immunofluorescence images of serial sections of the ESP-initiated lesion co-stained with DAPI and antibodies against αSMA and hCD31. Note that ESP-initiated vessel-like structures positive for hCD31 and Vm were surrounded by muscle-like layers positive for αSMA and Vm. Bars, 100 μm. (**E**) Immunofluorescence images of the same lesion as (**A**) co-stained with DAPI and antibodies against CK and vimentin (Vm). Bars, 500 μm. The borders between the reconstituted tissue and the mouse kidney (MK) are indicated by the dotted lines. (**F**) Immunofluorescence images of the ESP-initiated lesion co-stained with DAPI and antibodies against human CD31 (hCD31) and Vm in NOG mice. Yellow arrowheads indicate hCD31-positive cells. Bars, 200 μm. (**G**) Immunofluorescence images of the ESP-initiated lesion co-stained with DAPI and antibodies against PR and Vm. Bars, 50 μm.
doi:10.1371/journal.pone.0010387.g002

(Figure 2F). Indeed, out of the 24 mice xenotransplanted with ESP, only two mice displayed reconstitution of endometrium-like tissue with a glandular structure, which we designated as "glandular type" (Figure 2C).

Growth and differentiation potentials of ESP and EMP cells

To further characterize ESP cells *in vitro*, we attempted to cultivate and expand the cells using conventional media supplemented with various hormones and/or growth factors; however, ESP cells alone never proliferated efficiently. Furthermore, while SP cells derived from human myometrium are able to proliferate preferentially under hypoxic condition [14], ESP cells were not. When cultured together, however, ESP and EMP cells readily proliferated *in vitro* in conventional media (Figure 3A), suggesting that cell-to-cell interactions and/or EMP cell-derived secretory factors may be a prerequisite for the activation of ESP cells.

In vivo migratory and angiogenic potentials of freshly isolated ESP cells (Figure 2C, 2D, and S1) suggest that they may have endothelial cell (EC)-like properties. Therefore, we tested several media customized for EC culture and finally found that ESP cells were able to proliferate in EGM-2MV in the absence of EMP (Figure 3B and 3C).

A clonogenic assay using EGM-2MV medium revealed that the cloning efficiency of ESP cells ($0.143 \pm 0.008\%$ [mean ± SEM, $n = 5$]) was significantly less than that of EMP cells ($0.511 \pm 0.054\%$ [mean ± SEM, $n = 4$])(Figure 3B). It has been reported that SP cells isolated from the human endometrium, myometrium and other types of tissues are in the G_0 phase of the cell cycle [6,14,16,17]. Given the quiescence of ESP cells [6,14,16,17], it is conceivable that our clonogenic assay using short-term (14 days) culture may detect the clonogenic activities of committed progenitors (perhaps stromal progenitors) in EMP cells, but not those of the endometrial stem cells and/or the most primitive progenitors in ESP cells. Elucidation of the precise mechanism(s) awaits further studies.

Immunofluorescence studies revealed three distinct types of colonies in the ESP cultures. The most frequent type was comprised of fibroblastoid stromal cells positive for CD13, an endometrial stromal cell marker (Figure 3C). The second most frequent type displayed a cobblestone appearance and was comprised of CD31-positive cells (Figure 3C), which indicated similarities between these cells and late outgrowing endothelial progenitor cells (EPCs) [18]. The least frequent type of colony formed small nests that were comprised of epithelial cells positive for CK, an endometrial epithelial marker (Figure 3C). In contrast to ESP cultures, most of the colonies in the EMP cultures were comprised of CD13-positive fibroblastic stromal cells (Figure 3C). Therefore, ESP cells and not EMP cells possessed the stem/progenitor cell characteristic of generating many lineages of cell types within the tissue in which they are located.

Subpopulations of ESP cells and their morphology, surface markers, and localization in the human endometrium

To further characterize ESP cells, gene expression analysis of ESP and EMP cells was performed using RT-PCR. ESP cells preferentially expressed mRNA of universally SP-associated markers, ABCG2 and multidrug resistance 1 [15,19]. ESP cells also expressed CD31, CD34 and KDR (Figure 4A), consistent with the EC-like properties of ESP cells. Interestingly, estrogen receptor (ER) β was abundant in ESP, while neither ERα nor PR was detected. ERβ is also preferentially expressed in the vascular endothelial cells of the human endometrium [20]. The expression of these markers in ESP cells did not change after two weeks of culture (Figure 4A), except for a slight increase and decrease in ERα and ERβ mRNA expression, respectively. This indicates that EGM-2MV medium has the potential to maintain the undifferentiated status of ESP.

Flow cytometric analysis of ESP cells from whole endometrium stained with CD31, CD34, and CD144 antibodies, revealing that ESP cells preferentially expressed these EC-associated markers (Figure 4B). Thus, the expression pattern of the surface markers, the cobblestone-like cell morphology in EGM-2MV medium and the *in vivo* capacity to preferentially give rise to ECs (Figure 3C, 3D and 3F) are all properties of ESP cells that are shared with EC or EPC. In terms of hematopoietic or mesenchymal stem cell markers, our flow cytometric data showed that some SP cells express CD34, CD90, CD105 or CD146, but do not CD133 (Figure S2), and ESP is not a homogeneous population.

Since ESP cells, like other SP cells, expressed the SP-specific marker ABCG2 [15], we performed immunofluorescence staining of human cycling endometrium using ABCG2 antibody to address

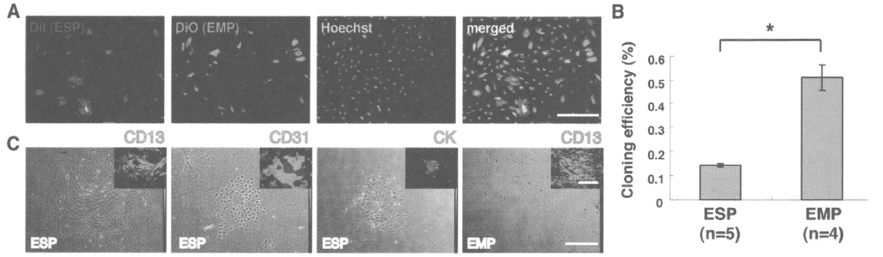


Figure 3. Growth and differentiation potentials of ESP and EMP cells. (**A**) Fluorescence images of DiI-labeled ESP cells co-cultured with DiO-labeled EMP cells following Hoechst DNA dye staining. Bars, 200 μm. (**B**) Cloning efficiencies of ESP cells and EMP cells in EGM-2MV medium. * $P<0.001$. Each bar indicates the mean ± SEM. $N \geq 4$. (**C**) Phase contrast micrographs and fluorescence images (insets) of colonies generated from ESP and EMP cultures. The ESP cells and EMP cells were separately seeded at a clonal density, cultured in EGM-2MV medium for two weeks, and subjected to immunofluorescence studies using antibodies against the indicated markers. CK, cytokeratin. Bars, 500 μm.
doi:10.1371/journal.pone.0010387.g003

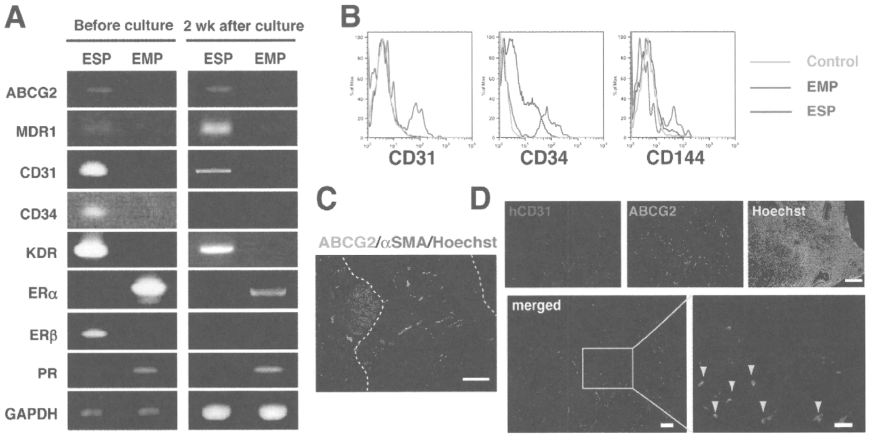


Figure 4. Subpopulations of ESP cells: surface markers, and localization in human endometrium. (**A**) RT-PCR analyses of various transcripts in ESP and EMP cells before and after two weeks of culture. MDR1, multidrug resistance 1; KDR, kinase insert domain-containing receptor. (**B**) Flow cytometric analysis of ESP and EMP cells immunostained with isotypic control antibodies or antibodies against endothelial cell surface markers (CD31, CD34, and CD144). (**C**) An immunofluorescence merged image of human endometrium co-stained with Hoechst and antibodies against ABCG2 and αSMA. White and yellow dotted lines indicate the endometrium-myometrium junction and the luminal surface of the uterine epithelium, respectively. Bars, 200 μm. (**D**) Immunofluorescence images of human endometrium co-stained with Hoechst and antibodies against hCD31 and ABCG2. A small box marks a region shown at a higher magnification in the adjacent panel as indicated. Yellow arrowheads indicate endothelial cells doubly positive for hCD31 and ABCG2. Bars, 200 μm (upper), 100 μm (lower left) and 50 μm (lower right).
doi:10.1371/journal.pone.0010387.g004

the tissue localization of ESP cells. The putative endometrial stem/progenitor cells are believed to reside in the *basalis* layer of the human endometrium. Given that the ESP contains candidate endometrial stem/progenitor cells, we anticipated $ABCG2^+$ cells might be predominantly located in the *basalis* layer. Unexpectedly, however, $ABCG2^+$ cells were evenly distributed across both the *functionalis* and *basalis* layers of the endometrium (Figure 4C).

Taking into account the expression of CD31 in the ESP, we performed double immunofluorescence staining using CD31 and ABCG2 antibodies to examine the localization of $CD31^+$ ESP cells in human cycling endometrium. We found that $CD31^+ABCG2^+$ cells, presumably containing $CD31^+$ ESP cells, were preferentially located in small capillaries, rather than large vessels (Figure 4D). Again, $CD31^+ABCG2^+$ cells were present throughout the *functionalis* and *basalis* layers.

Discussion

Human and primate endometrium regenerates from the lower *basalis* layer, a germinal compartment that persists after menstruation to give rise to the new upper *functionalis* layer [2,21–23]. The surface epithelium develops primarily through the proliferation of epithelial cells from the tips of the gland stumps [24,25]. The findings presented here strongly support the idea that the basalis of the endometrium harbors stem/progenitor cells responsible for endometrial regeneration during menses as well as after parturition in both women and menstruating non-human primates [23]. It remains possible, however, that endometrial stem/progenitor cells also exist in the *functionalis* of the endometrium. Indeed, the $ABCG2^+$ population, which presumably includes ESP cells having endometrial stem cell-like properties (present study and [5]), is localized exclusively in the endothelium of both the functional and basal layers of the human endometrium (Figure 3 and [6]). A relatively small number of dispersed human endometrial cells (mainly derived from the *functionalis* layer of the endometrium) containing ESP cells can generate functional endometrial tissue comprising glands, stroma, immune cells and vascular components when they are transplanted under the kidney capsule of severely immunodeficient mice [1]. Mesenchymal stem-like cells expressing both CD146 and PDGF-Rβ are located perivascularly in the *functionalis* and *basalis* layers of the human endometrium [7]. Very recently, Garry *et al.* demonstrated that endometrial surface epithelial regeneration takes place as a consequence of cellular differentiation from stromal cells and not by direct extension from the residual basal epithelial glands [26]. These findings collectively suggest that ESP cells present in the vascular endothelium are one of the most likely candidates for endometrial stem/progenitor cells that may reside not only in the *basalis* but also in the *functionalis* endometrium.

The percentage of SP cells derived from cultured epithelial-enriched fraction was significantly greater during menstruation than at any other cycle stage [5], whereas the rate of SP cells freshly isolated from endometrial samples was significantly greater in the proliferative phase than in the secretory phase [6]. In this study we showed that the proportion of ESP cells to the EMP + ERP fraction freshly isolated from human endometria was the highest at the early proliferative phase among all the phases of the menstrual cycle, which is in agreement with previous report [6], and it decreased gradually until its nadir in the late secretory phase. Given the stem cell-like properties of ESP cells as presented

here and reported elsewhere [5,6], it is tempting to speculate that ESP cells may generate committed progenitor cells through asymmetrical cell division, which, in turn, may lose the SP phenotype, further behave as transient amplifying cells, and eventually propagate through symmetrical cell division. In this context, the gross number of ESP cells may be almost unchanged, but the number of non-SP cells (EMP + ERP cells) present particularly in the *functionalis* endometrium may increase, presumably resulting in the decline in the proportion of ESP cells from menstruation towards the late secretory phase.

There are several explanations for the low efficiency of reconstitution. First, the local environment at the xenotransplantation site may lack necessary factors for the regeneration of the entire endometrium. Second, ESP cells may require a specific "niche" provided by other endometrial cell components to reconstitute the entire endometrium *in vivo* as well as *in vitro* culture. Successful proliferation of ESP cells in the presence of EMP cells in conventional media (Figure 3A) suggests that EMP cells may provide a "niche" appropriate for activation of ESP cells. Moreover, successful proliferation of ESP cells alone in EGM-2MV medium prompts us to postulate that EMP cells alone and/or cell-to-cell interaction between ESP and EMP cells may produce bioactive substances such as EGF, VEGF, bFGF, and/or insulin-like growth factor-I that EGM-2MV medium contains. Microenvironments under the kidney capsule of NOG mice may also provide some but not sufficient "niche", which may be at least in part attributed to low efficiency of reconstitution of organized endometrial tissue from ESP cells. We have previously demonstrated that the single cell suspension without any cell selection which consisted of ESP cells and non-SP cells could reconstitute well-organized endometrial tissue at almost 100% frequency [1], which is much greater than the rate of ESP cells alone (8%). These findings suggest that EMP cells are required as a "niche" provider for *in vivo* tissue reconstitution but EMP cells alone may not be able to generate the organized endometrial tissues *in vivo*. Third, ESP cells might not contain a sufficient number of stem/progenitor cells for generating glandular cells. In this study, we collected endometrial tissues by scraping strongly the uterine cavity with the back edge of a scalpel. A portion of the endometrium, particularly the deep *basalis* layer, however, penetrates into the myometrium where it will not be collected by scraping (Figure S3). Thus, it is possible that some of the ESP cells present in the deep *basalis* layer may not be included in the starting endometrial materials in this study.

In this study, we have shown that at least some ESP cells were localized to the endometrial endothelial wall, predominantly expressed several EC markers, preferentially proliferated and differentiated *in vitro* in an EC-specific medium, and displayed high migratory and angiogenic potential. These results suggest that ESP cells have endometrial stem cell-like properties as well as EC or EPC-like characteristics. EPCs are believed to be derived from the bone marrow and to home to sites of neovascularization and neoendothelialization where they differentiate into ECs [27,28]. This raises the possibility that ESP may have originated from bone marrow stromal cells. Indeed, bone marrow-derived EPCs contribute to the formation of new blood vessels in human and mouse endometrium [29,30]. Furthermore, bone-marrow derived cells give rise to uterine epithelial cells in humans [31] and mice [32,33], although the identity of these cells remains unclear. Based on the present results, we speculate that ESP represents one such candidate population.

In view of these findings, we here propose a single model for ESP-driven endometrial regeneration and establishment of endometriosis (Figure 5). In this model, ESP cells, perhaps

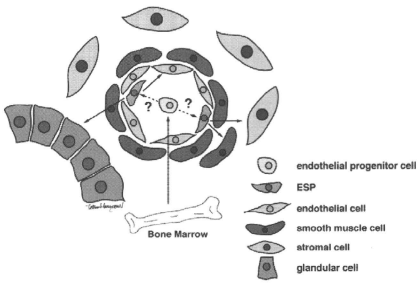


Figure 5. Proposed model for ESP-driven endometrial regeneration and the establishment and progression of endometriosis.
doi:10.1371/journal.pone.0010387.g005

ultimately derived from the bone marrow, mainly reside in vascular endothelial walls and/or perivascular regions. Importantly, these ESP cells are present not only in the *basalis* but also in the *functionalis* endometrium. These cells, therefore, might be contained within the sloughed endometrium shed at menstruation. They might then implant onto the surface of ectopic sites such as the peritoneum through retrograde menstruation. Furthermore, some of these *functionalis* layer-derived ESP cells might remain in the uterine cavity after menstruation and implant again (reimplant) onto the deconstructed eutopic endometrium. In both eutopic and ectopic implantation, endothelial ESPs might give rise to various endometrial cell components in the process of ESP-driven angiogenesis. Our eutopic reimplantation hypothesis does not contradict the current paradigm but rather provides an additional mechanism for endometrial regeneration. We describe previously that a certain type of cells in endometrium could migrate, invade, form chimeric vasculature in the host kidney of NOG mouse and establish the functional circulatory system[1]. In terms of the ability to invade into kidney parenchyma, these cells could be SP cells. From this point, their ability may be crucial for establishment and development endometriosis, because the angiogenesis is absolutely required for maintenance of endometriotic lesion. In support of this idea, a stem cell theory of the pathogenesis of endometriosis has been recently emerged [34], postulating that endometrial stem/progenitor cells may function in the development of endometriosis. Our proposed new model suggests that only a small population of endometrial cells, specifically ESP cells, has the potential to generate endometriotic lesions through their unique migratory and angiogenic activities and provides a proving ground of anti-angiogenic therapy recently proposed as a potential treatment for endometriosis [35–37].

We have shown that ESP cells, but not EMP cells, express ERβ but do not express ERα or PR. A similar expression pattern is observed in endometrial vascular ECs which express ERβ, but neither ERα nor PR [20]. Those findings further substantiate our present results that ESP cells have EC-like properties and reside in the endometrial endothelium. Finally, the expression level of ERβ is strikingly higher than those of ERα and PR in endometriotic lesions [38], implicating ERβ-positive ESP cells in the pathogenesis of endometriosis.

In summary, we have demonstrated that undifferentiated ESP cells are present in human cycling endometrium. Purified ESP cells, but not EMP cells, contain putative endometrial stem/

progenitor cells with potentials for differentiation into multiple types of endometrial cells *in vitro* and *in vivo*. In our hands, ESP cells were not able to proliferate from a single cell *in vitro*. Without single cell analyses, it remains uncertain whether individual ESP cells posses multi-lineage differentiation potential at the clonal level or, instead, the cells consists of a mixed population of progenitors or stem cells. The study of the ESP, however, will improve the understanding of endometrial physiology and provide insight into the pathogenesis and treatment of endometrium-derived diseases such as endometriosis.

Materials and Methods

Detailed protocols can be found in Methods S1.

Tissue collection

Endometrial specimens (n = 78) were collected from women with normal menstrual cycles undergoing total abdominal hysterectomy for benign gynecological diseases, or cervical carcinoma *in situ*. Written informed consent was obtained from each patient and the use of these human specimens was approved by the Keio University Ethics Committee.

Isolation and flow cytometric analysis of ESP and EMP cells

Endometrial specimens were separated and dissociated into endometrial stromal and glandular epithelial single cell fractions as described previously [1]. Stromal-enriched and epithelial-enriched fractions were washed in calcium- and magnesium-free HBSS supplemented with 2% FBS, 10 mM HEPES buffer, and 1% penicillin-streptomycin ($HBSS^+$) and suspended at 2×10^6 cells/mL in $HBSS^+$ and stained with 5.0 μg/mL Hoechst33342 (Sigma Chemical, St. Louis, MO) for 90 min at 37°C, as described previously [12,13]. Fluorescein isothiocyanate, phycoerythrin or allophycocyanin-conjugated antibodies for flow cytometry and propidium iodide were simultaneously added to Hoechst-stained cells suspended in $HBSS^+$. Cells were incubated on ice for 30 minutes, pelleted, and washed with $HBSS^+$. The antibodies we used are listed in Table S1. Flow cytometric analysis and cell sorting were performed as described in *SI Methods*. After collecting 1×10^5 events, the SP population was defined as previously reported [13].

Co-culture of SP and MP cells

To track the fate of SP cells in co-culture with MP cells, the SP and MP cells were labeled with two different fluorescent dyes-Vybrant DiO (green fluorochrome) and Vybrant DiI (red fluorochrome) (Molecular Probes, Eugene, OR). These reagents allow two-color labeling of cell populations for identification after mixing and co-culture. DiI-labeled SP cells and DiO-labeled MP cells were mixed at a ratio of 1:1 and co-cultured in DMEM containing 1% antibiotic-antimycotic, and 10% FBS.

Cell culture and determination of cloning efficiency

SP and MP cells were separately seeded at a clonal density of 400 cells/cm^2 in 35 mm dishes and cultured in EGM-2MV medium (Cambrex, Walkersville, MD). After 14 days of culture, clusters of cells were considered colonies when they were visible macroscopically and had more than 50 cells. Colonies were counted and the cloning efficiency (CE) was determined from the formula: CE (%) = (number of colonies/number of cells seeded) ×100.

RT-PCR

Total RNA was extracted from cell cultures and subjected to RT-PCR. First-strand cDNA was synthesized and amplified using specific PCR primers (Table S2). The PCR products were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining with UV light illumination.

Xenotransplantation and hormonal treatment

The same numbers of SP and MP cells (10^4 to 10^5 cells), freshly isolated from human endometria, were immediately transplanted under the kidney capsules of NOG mice as described previously [1].

Histology and immunohistochemistry

H&E-staining and immunofluorescence analyses were performed on culture dishes or cryosections derived from kidneys transplanted with ESP or EMP, that were air-dried, washed, and fixed. After permeabilization and blocking, tissue sections were incubated with the pretitrated primary antibodies listed in Tables S1 and S3. For indirect fluorescence staining, the first antibodies were visualized by incubation with secondary antibodies conjugated with Alexa Fluor 488 (green) or 568 (red) (Molecular Probes). We classified the histological feature of the reconstituted tissue and its adjacent transplantation site into four subtypes (i.e., glandular, vascular endothelial, migrating endothelial, and stromal), based on their dominant type (Figure 2C). The reconstitution rate of ESP and EMP cells was determined by the following formula: Reconstitution rate (%) = (number of a corresponding subtype/number of transplanted kidneys) ×100. Images were collected as described in *SI Methods*.

Statistics

Results are expressed as means ± SEM. Comparisons among the SP rates for each of the four phases were made with a Tukey test and the others were done using the unpaired Student's t test. P values less than 0.05 were considered statistically significant.

Supporting Information

Figure S1 Expression of endothelial and stromal cell markers in human-derived cells present around the ESP-initiated lesion. Immunofluorescence images of the ESP-initiated lesion in NOG mouse kidney co-stained with DAPI and antibodies against Vm and hCD31 (A) or antibodies against Vm and CD13 (B). Bars, 100 μm.
Found at: doi:10.1371/journal.pone.0010387.s001 (4.16 MB TIF)

Figure S2 Expression of hematopoietic stem cell marker and mesenchymal stem cell marker on ESP cells. Flow cytometric analysis of ESP cells stained with antibodies against hematopoietic stem cell markers (CD34 and CD133) and mesenchymal stem cell markers (CD90, CD105 and CD146).
Found at: doi:10.1371/journal.pone.0010387.s002 (3.15 MB TIF)

Figure S3 Remaining basalis layer of endometrium after endometrial tissue has been scraped off. Histological and immunofluorescence images of the uterine interface between the endometrium and myometrium stained with H&E or antibodies against CK and αSMA. Bars, 500 μm. Black and yellow arrowheads indicate endometrial glands present adjacent to or inside the myometrium.
Found at: doi:10.1371/journal.pone.0010387.s003 (0.96 MB TIF)

Table S1 List of antibodies used for flow cytometric analysis.
Found at: doi:10.1371/journal.pone.0010387.s004 (0.03 MB DOC)

Table S2 Sequences of the primers used for detection of various genes.
Found at: doi:10.1371/journal.pone.0010387.s005 (0.04 MB DOC)

Table S3 List of antibodies used for immunofluorescence staining.
Found at: doi:10.1371/journal.pone.0010387.s006 (0.04 MB DOC)

Methods S1
Found at: doi:10.1371/journal.pone.0010387.s007 (0.07 MB DOC)

References

1. Masuda H, Maruyama T, Hiratsu E, Yamane J, Iwanami A, et al. (2007) Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/γ_c^{null} immunodeficient mice. Proc Natl Acad Sci U S A 104: 1925–1930.
2. Padykula HA (1991) Regeneration in the primate uterus: the role of stem cells. Ann N Y Acad Sci 622: 47–56.
3. Gargett CE, Chan RW, Schwab KE (2008) Hormone and growth factor signaling in endometrial renewal: role of stem/progenitor cells. Mol Cell Endocrinol 288: 22–29.
4. Chan RW, Schwab KE, Gargett CE (2004) Clonogenicity of human endometrial epithelial and stromal cells. Biol Reprod 70: 1738–1750.
5. Kato K, Yoshimoto M, Kato K, Adachi S, Yamayoshi A, et al. (2007) Characterization of side-population cells in human normal endometrium. Hum Reprod 22: 1214–1223.
6. Tsuji S, Yoshimoto M, Takahashi K, Noda Y, Nakahata T, et al. (2008) Side population cells contribute to the genesis of human endometrium. Fertil Steril 90: 1528–1537.
7. Schwab KE, Gargett CE (2007) Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Hum Reprod 22: 2903–2911.
8. Dimitrov R, Timeva T, Kyurkchiev D, Stamenova M, Shterev A, et al. (2008) Characterization of clonogenic stromal cells isolated from human endometrium. Reproduction 135: 551–558.
9. Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. Stem Cells 24: 3–12.
10. Tomita Y, Matsumura K, Wakamatsu Y, Matsuzaki Y, Shibuya I, et al. (2005) Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. The Journal of cell biology 170: 1135–1146.
11. Hishikawa K, Marumo T, Miura S, Nakanishi A, Matsuzaki Y, et al. (2005) Musculin/MyoR is expressed in kidney side population cells and can regulate their function. The Journal of cell biology 169: 921–928.
12. Matsuzaki Y, Kinjo K, Mulligan RC, Okano H (2004) Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. Immunity 20: 87–93.
13. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183: 1797–1806.
14. Ono M, Maruyama T, Masuda H, Kajitani T, Nagashima T, et al. (2007) Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells. Proc Natl Acad Sci U S A 104: 18700–18705.
15. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, et al. (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nature medicine 7: 1028–1034.
16. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, et al. (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118: 149–161.
17. Umemoto T, Yamato M, Nishida K, Yang J, Tano Y, et al. (2006) Limbal Epithelial Side-Population Cells Have Stem Cell-Like Properties, Including Quiescent State. Stem Cells 24: 86–94.
18. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, et al. (2004) Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 24: 288–293.
19. Zhou S, Zong Y, Lu T, Sorrentino BP (2003) Hematopoietic cells from mice that are deficient in both Bcrp1/Abcg2 and Mdr1a/1b develop normally but are sensitized to mitoxantrone. BioTechniques 35: 1248–1252.
20. Critchley HO, Brenner RM, Henderson TA, Williams K, Nayak NR, et al. (2001) Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium. J Clin Endocrinol Metab 86: 1370–1378.
21. Prianishnikov VA (1978) On the concept of stem cell and a model of functional-morphological structure of the endometrium. Contraception 18: 213–223.
22. Padykula HA, Coles LG, Okulicz WC, Rapaport SI, McCracken JA, et al. (1989) The basalis of the primate endometrium: a bifunctional germinal compartment. Biol Reprod 40: 681–690.
23. Okulicz WC (2002) Regeneration. In: Glasser SR, Aplin JD, Giudice LC, Tabibzadeh S, eds. The Endometrium. London: Taylor & Francis Inc. pp 110–120.
24. Ferenczy A (1976) Studies on the cytodynamics of human endometrial regeneration. II. Transmission electron microscopy and histochemistry. Am J Obstet Gynecol 124: 582–595.
25. Salamonsen LA (2003) Tissue injury and repair in the female human reproductive tract. Reproduction 125: 301–311.
26. Garry R, Hart R, Karthigasu KA, Burke C (2009) A re-appraisal of the morphological changes within the endometrium during menstruation: a hysteroscopic, histological and scanning electron microscopic study. Hum Reprod 24: 1393–1401.
27. Urbich C, Dimmeler S (2004) Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 95: 343–353.
28. Timmermans F, Plum J, Yoder MC, Ingram DA, Vandekerckhove B, et al. (2008) Endothelial progenitor cells: Identity defined? J Cell Mol Med.
29. Masuda H, Kalka C, Takahashi T, Yoshida M, Wada M, et al. (2007) Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis. Circ Res 101: 598–606.
30. Mints M, Jansson M, Sadeghi B, Westgren M, Uzunel M, et al. (2008) Endometrial endothelial cells are derived from donor stem cells in a bone marrow transplant recipient. Hum Reprod 23: 139–143.
31. Taylor HS (2004) Endometrial cells derived from donor stem cells in bone marrow transplant recipients. Jama 292: 81–85.
32. Du H, Taylor HS (2007) Contribution of bone marrow-derived stem cells to endometrium and endometriosis. Stem Cells 25: 2082–2086.
33. Bratincsak A, Brownstein MJ, Cassiani-Ingoni R, Pastorino S, Szalayova I, et al. (2007) CD45-positive blood cells give rise to uterine epithelial cells in mice. Stem Cells 25: 2820–2826.
34. Sasson IE, Taylor HS (2008) Stem cells and the pathogenesis of endometriosis. Ann N Y Acad Sci 1127: 106–115.
35. Dabrosin C, Gyorffy S, Margetts P, Ross C, Gauldie J (2002) Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. Am J Pathol 161: 909–918.
36. Hull ML, Charnock-Jones DS, Chan CL, Bruner-Tran KL, Osteen KG, et al. (2003) Antiangiogenic agents are effective inhibitors of endometriosis. J Clin Endocrinol Metab 88: 2889–2899.
37. Nap AW, Griffioen AW, Dunselman GA, Bouma-Ter Steege JC, Thijssen VL, et al. (2004) Antiangiogenesis therapy for endometriosis. J Clin Endocrinol Metab 89: 1089–1095.
38. Bulun SE (2009) Endometriosis. N Engl J Med 360: 268–279.

Acknowledgments

We thank Tetsuro Tamaki for advice on xenotransplantation; Hiroko Kouike and Rei Sakurai for technical assistance; and Shino Kuwabara and Rika Shibata for secretarial assistance.

Author Contributions

Conceived and designed the experiments: HM YM TM HO. Performed the experiments: HM YM EH MO TN TK TA HO HU TM. Analyzed the data: HM TM. Contributed reagents/materials/analysis tools: HM YM HA MI TM HO. Wrote the paper: HM YM YY TM HO.

doi:10.1111/j.1447-0756.2010.01473.x J. Obstet. Gynaecol. Res. 2011

Change of salivary stress marker concentrations during pregnancy: Maternal depressive status suppress changes of those levels

Hiroaki Tsubouchi[1], Yuichiro Nakai[2], Masahiro Toda[3], Kanehisa Morimoto[4], Yang Sil Chang[2], Norichika Ushioda[2], Shoji Kaku[2], Takafumi Nakamura[2], Tadashi Kimura[5] and Koichiro Shimoya[2]

[1]Department of Obstetrics and Gynecology, Aizenbashi Hospital, Osaka, [2]Department of Obstetrics and Gynecology, Kawasaki Medical School, Kurashiki, [3]Department of Pharmacology, Osaka Dental University, Hirakata, and Departments of [4]Social and Environmental Medicine and [5]Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan

Abstract

Aim: The aim of the present study was to show changes in salivary cortisol and chromogranin A/protein concentrations as stress markers during pregnancy and to clarify the effect of chronic stress on stress markers.

Material and Methods: Salivary samples were collected from 69 pregnant women during pregnancy. Salivary cortisol levels and chromogranin A/protein titers were determined. We surveyed the women's chronic stress using the Zung self-rating depression scale and General Health Questionnaire-28.

Results: Cortisol levels in the saliva of pregnant women showed biphasic change during pregnancy. Chromogranin A/protein levels in the saliva of pregnant women increased in the second and the early third trimesters and decreased to the puerperal period. Salivary cortisol concentrations of the chronic high stress group were significantly lower compared with those of the normal group. Salivary chromogranin A/protein concentrations of the chronic high stress group were also significantly lower than those of the normal group.

Conclusion: The titration of salivary cortisol concentrations and chromogranin A/protein levels is a useful tool to determine maternal stress levels. The elevation of cortisol and chromogranin A/protein in the saliva was suppressed in the chronic high stress group during pregnancy.

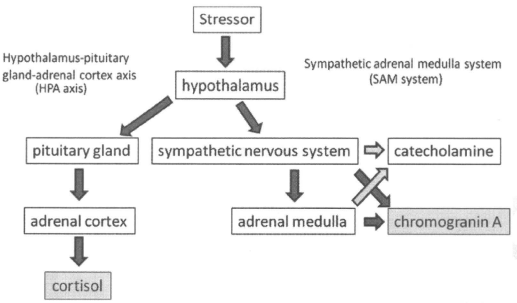
Key words: chromogranin A, depressive status, maternal stress, pregnancy, salivary cortisol.

Introduction

Psycho-mental health potentials were evaluated by stress-related hormonal levels. Data indicate that individuals with good lifestyles showed much younger health ages calculated based on health check-up data, and lower risks for developing lifestyle-related diseases than those with poor lifestyles. Comprehensive health potentials were significantly lower in poor-lifestyle individuals than those with a good lifestyle.[1]

Maternal psycho-mental stress has an adverse effect on pregnancy, such as preterm labor,[2] intrauterine growth restriction[3] and fetal anomalies.[4] Recently, it was reported that maternal cortisol levels in the urine were associated with early pregnancy loss.[5] As shown in Figure 1, the stressor has an effect on the hypothalamus and affects both the hypothalamus-pituitary gland-adrenal cortex axis and sympathetic adrenal medulla system. The markers secreted in the saliva are useful because they are easy to measure without

Received: ••.
Accepted: ••.
Reprint request to: Dr Koichiro Shimoya, Department of Obstetrics and Gynecology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan. Email: shimoya@med.kawasaki-m.ac.jp
The authors indicate no potential conflicts of interest.



Colour

Figure 1 Mechanism of induction of cortisol and chromogranin A by stressor.

causing sampling stress or pain. Evaluation of physiological stress markers in the saliva, such as cortisol and catecholamine, is a very useful method for objectively assessing stress. Salivary cortisol is often used as a stress index because its measurement has various advantages, such as non-invasive collection procedure. However, the measurement of salivary catecholamine is difficult because of its low concentration and rapid degradation.[6] Chromogranin A (CgA) is a good marker of psychological stress response, because it reflects only psychological stress, not physiological stress.[7,8] CgA is an acidic-soluble glycoprotein and its concentration can be measured in the saliva. It is known to localize in the secretory granules of a wide variety of endocrine and neuronal tissues.[9] The level of salivary CgA provides a sensitive and reliable index for evaluating psychological stress.[7] CgA is considered to be a substitute for catecholamine as CgA and catecholamine are co-released into the extracellular environment. Therefore, it is considered to be a measure of the activity of the sympathetic/adrenomedullary system, and salivary CgA has begun to be used as a stress marker.[6] We reported that salivary cortisol levels were significantly reduced after coffee intake, but not salivary chromogranin A concentration during pregnancy.[10] Nierop *et al.* demonstrated that salivary cortisol recovery was significantly prolonged in second-trimester pregnant women and prolonged cortisol recovery during the beginning of second-trimester pregnancy might be associated with vulnerability to stress-related pregnancy complications during this period of time.[11] The reaction of stress response during pregnancy was different from subjects of non-pregnant status. However, there is little information regarding the detailed changes of salivary stress markers during pregnancy.

The aim of the present study was to show changes in salivary cortisol and CgA concentrations as stress markers during pregnancy and to clarify the difference of changes in these stress markers during pregnancy between chronic high stress and low stress groups.

Material and Methods

Samples

Saliva samples were obtained at 09.00 to 13.00 hours in the first trimester (10–12 w), second trimester (20–22 w), early third trimester (30–32 w), late third trimester (37–39 w), and puerperal period (1 month after delivery) from 69 normal pregnant women at Osaka University Hospital. Their average age was 32 years (20–40 years). Thirty one of the women were primipara. 34 had only one child and four had two children among the multiparas. We excluded patients who were taking steroid orally, or had complications or a fetus with an anomaly. The study was approved by the local ethics committee of the Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine. Informed consent was obtained from each patient. Examinations were performed from 10.00 to 11.00 hours, starting more than 2 h after consumption of the morning meal, because no food should be consumed for at least 90 min before salivary sampling for cortisol determination.[12] All saliva samplings were performed in the hospital under the researcher's guidance.