Table 2
Evaluation of PTEN, p-Akt, p53, ER and PgR expressions in pre- and under-MPA samples from 31 cases with CAH or IaG1

Case	Pre-MPA IHC					Under-MPA IHC					Duration of MPA[c] (months)
	PTEN	p-Akt	p53	ER	PgR	PTEN	p-Akt	p53	ER	PgR	
1	0 + 1	2	1	1	2	1 + 2	1	1	3	3	2.1
2	1	2	0 + 1	2	2	1 + 2	0	1	2	2	2.2
3	1	2	1	2	3	1	0	1	2	2	2.1
4	1 + 2	2	1	2	2	...[a]	...	...	...	...	...
5	2	1	0	1	2	...[a]	...	...	...	...	...
6	0 + 1	1	1	0 + 2	0	...[a]	...	...	...	...	...
7	1	2	1	1	1	1	1	1	3	3	3.2
8	0	1	2	3	3	0 + 2	2	1	3	3	6.0
9	1 + 2	2	1	3	3	1 + 2	1	1 + 2	3	3	2.1
10	1	3	1	2	3	...[a]	...	...	...	...	...
11	2	2	1	1 + 2	2	...[a]	...	...	...	...	...
12	1	3	1 + 2	1	3	1	2	1	3	3	2.3
13	1	3	1	1	3	1	1	1	2	2	2.5
14	2 + 3	2	1 + 2	1	0 + 2	...[a]	...	...	...	...	...
15	0 + 1	2	1	1	2	...[a]	...	...	...	...	...
16	0 + 1	2	1	2	3	1 + 2	1	2	2	1	6.0
17	1	1	0	1	2 + 3	0	0	2	2	2	2.3
18	0	2	1	1	3	0	2	0	2	3	1.3
19	0 + 1 + 2	1	1	2	2	0 + 1	1	1	3	3	2.1
20	0	1	1 + 2	1	2	0 + 1	1	1	2	2	2.5
21	1 + 2	2	1 + 2	2	3	...[a]	...	...	...	...	...
22	0 + 1 + 2	1	2	2	3	2	1	1	1	2	2.0
23	0 + 1	2	1	2	0 + 1	...[a]	...	...	...	...	...
24	1	0	0	1	2	...[b]	...	...	...	...	...
25	0	2	1	1	2	1	1	1	2	3	4.0
26	1	2	1 + 2	1 + 2	2 + 3	0 + 1	1	1	3	2	2.3
27	2	3	1	3	3	...[a]	...	...	...	...	...
28	1 + 2 + 3	2	1 + 2	1 + 2	1	0	2	1 + 2	3	3	3.0
29	0 + 1 + 2	3	1	3	2	0	1	1 + 2	3	3	2.5
30	1	1	1	2	3	1	1	1	3	3	2.4
31	1	3	1	2	0	...[a]	...	...	...	...	...

IHC score: 0, negative; 1, decreased; 2, equal; and 3, increased staining intensity compared to the corresponding normal endometrial epithelium. "0 + 1" means a heterogeneous staining pattern where tumor cells with negative and decreased staining are mixed.
[a] Not determined because no tumor cells were found in under-MPA samples due to complete response.
[b] Not determined because sample conditions were not informative.

endometrial tumors has not been clarified. It has been recently reported that the level of phospho-Akt is decreased in endometrial stromal cells undergoing progesterone-induced in vitro decidualization [24]. Marino et al. have reported that estradiol-induced Akt phosphorylation is modulated by PTEN via MAPK pathway [13]. Furthermore, a recent study by Guzeloglu-Kayisli et al. has proposed that estradiol may downregulate PTEN activity by increasing its phosphorylation, while progesterone is likely to regulate the PTEN pool by decreasing its phosphorylation and increasing its protein level in endometrial cells [14]. These published findings suggest that anti-tumor effect of progesterone on endometrial tumor may be mediated by upregulation of PTEN protein, which subsequently downregulates Akt phosphorylation. In fact, the current study has shown that phospho-Akt expression is significantly decreased by MPA treatment (Figs. 1C, D and 2). Our observation in vivo is also consistent with the recent report by Chen et al. that progesterone inhibits the PI3K/Akt/GSK3β/cyclin D1/pRb pathway to block uterine epithelial cell proliferation [25]. Furthermore, we have found that presence of PTEN-null tumor cells and weak phospho-Akt expression in pre-treatment specimens are both associated with a trend to undergo hysterectomy, and that combination of these two factors correlates with receiving hysterectomy with statistical significance (Table 3). Because undergoing

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Table 3
Associations between pathological/immunohistochemical variables and post-treatment hysterectomy after MPA therapy or pregnancy after infertility treatment

Variable	Hysterectomy		Pregnancy	
	No./Total (%)	p value[a]	No./Total (%)	p value[a]
Histology				
G1	7/19 (37)		3/11 (27)	
CAH	1/10 (10)	0.2	5/9 (56)	0.4
PTEN-null tumor cells[b,c]				
Present	5/12 (42)		ND	
Absent	3/17 (18)	0.2	ND	–
p-Akt[b]				
<2	5/9 (56)		ND	
2⩽	3/20 (15)	0.07	ND	–
PTEN-null or p-Akt < 2[b,c]				
Yes	7/16 (44)		ND	
No	1/13 (8)	0.04	ND	–
p53[b]				
2⩽	3/8 (38)		ND	
<2	5/21 (24)	0.6	ND	–
ER[b]				
2⩽	5/13 (39)		6/9 (67)	
<2	3/16 (19)	0.4	2/11 (18)	0.06
PgR[b]				
=3	3/11 (27)		5/8 (63)	
<3	5/18 (28)	1.0	3/12 (25)	0.2
ER ⩾ 2 and PgR = 3[b]				
Yes	3/8 (38)		5/6 (83)	
No	5/21 (24)	0.6	3/14 (21)	0.02

[a] p value was calculated by χ^2 test or Fisher's exact test, where appropriate.
[b] IHC score of each case is shown in Table 2.
[c] PTEN-null means IHC score including 0.

hysterectomy, which means no response to MPA or relapse of carcinoma after MPA in our treatment protocol, should represent the consequence of biological sensitivity of tumor to the treatment, these observations can be interpreted as follows; PTEN-null tumor cells can be thought to be resistant to PTEN-mediated apoptosis and/or growth arrest, and lack of activated Akt means that down-regulation of Akt has weak or no effect on the tumor cells. Interestingly, PTEN null and low phospho-Akt were found to mostly coexist in the same cells in the samples exhibiting both of these staining patterns (Fig. 1I and J), suggesting that another unknown alteration in some component of the PI3K pathway may be interrupting the activation of Akt by PTEN dysfunction in these tumor cells. Although our immunohistochemical analysis has shown that phosphorylation of Akt is significantly decreased by MPA administration, we have failed to detect increase of PTEN expression by the treatment. We speculate that it may be theoretically difficult to fully evaluate PTEN activation/inactivation only by immunohistochemical analysis, because PTEN inactivation includes genetic alteration such as missence mutations and post-translational modification such as phosphorylation of the C-terminal tail and oxidation of cysteine residues in the catalytic site, all of which cannot be detected by our immunohistochemistry [26,27]. Additionally, we previously demonstrated that different mutational sites in PTEN gene might have different degrees of PTEN dysfunction, causing different prognostic impacts in patients with endometrial carcinoma [8]. Moreover, Agrawal et al. have recently reported that different splice-site mutations of PTEN gene resulting in the deletion of different exons lead to distinct biochemical outcomes such as reduced protein and/or lipid phosphatase activities [28].

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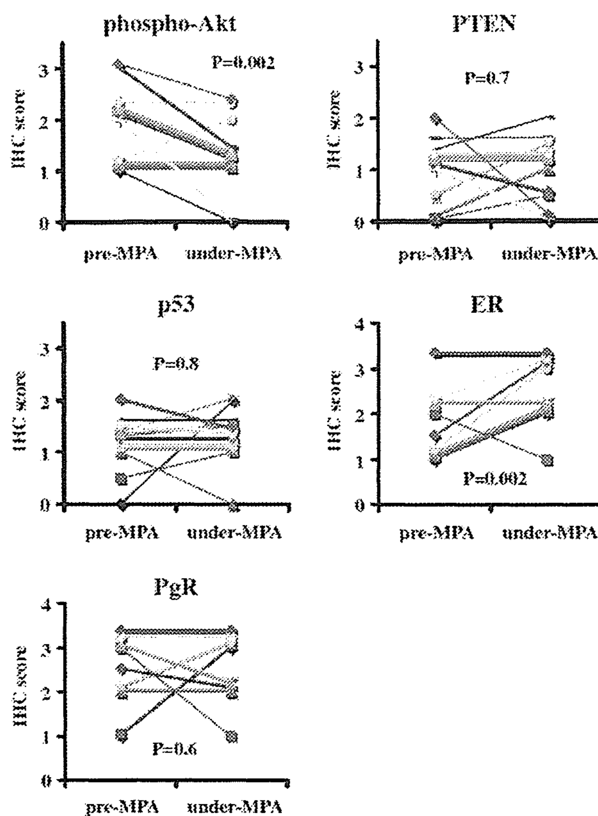

Fig. 2. Comparison of immunohistochemical stainings of p-Akt, PTEN, p53, ER and PgR in pre- and under-MPA samples from endometrial tumors ($n = 19$). IHC scores of each case are shown in Table 2. Representative score for cases with heterogenous staining pattern was calculated as the average of total scores. P value was calculated by Wilcoxon signed-ranks test.

We suspect that insufficient evaluation of PTEN dysfunction by immunohistochemistry may be also the reason why the presence of PTEN-null cells was not a statistically significant factor correlating with receiving hysterectomy in the current study.

Our study has examined only pre- and under-MPA treatment samples, but not post-treatment samples. In a recent study by Zheng et al., they have examined PTEN immunohistochemistry of pre and postprogestin-treated endometria and concluded that progestin therapy promotes involution of PTEN-null endometrial glands [2]. However, it can also be speculated that progestin treatment must have induced involution of not only PTEN-null glands but also PTEN-expressing glands, because the current study has demonstrated that there are 12 strongly phospho-Akt expressing cases without PTEN-null tumor cells which completely responded to MPA without resulting in hysterectomy (Table 1). It is more plausible to speculate that progestin induces apoptosis in all endometrial glands including neoplastic glands which have acquired PTEN inactivation, resulting in generation of normal glands without PTEN inactivation in post-MPA atrophic endometria. It is further suggested from our observations that a small number of PTEN-null or weak-phospho-Akt tumor cells, which are resistant to progestin, survive, resume proliferation after treatment, and progress to persistent disease.

Our immunohistochemical evaluation has found no significant difference in the staining intensity of p53 between pre- and under-MPA samples. However, Kester et al. have reported that prolonged progestin treatment induces the promoter of p21 through activation of p53 in human endometrial tumor cells, and that the activation of p53 by progestin may be caused by nuclear translocation of p53 [29]. Additionally, they have noted that p53 is mostly nuclear after 1 day of progestin treatment, but the difference is less pronounced for longer treatments. Because the duration of MPA treatment between pre- and under-MPA samplings in our study is 2.8 months on average (Table 2), that may be the reason why we did not observe difference in nuclear p53 expressions by MPA. It is possible that phosphorylation or acetylation of p53 is involved in its activation by long-term progestin treatment [30].

Infertility treatment after long duration of progestin therapy is occasionally very difficult because of poor response of hormonally ablated atrophic endometria. However, our data have shown that strong expression of both ER and PgR in tumor cells in pre-MPA samples is significantly associated with successful pregnancy after infertility treatment following CR to MPA. Although we also examined expressions of ER and PgR in endometrial stroma, no association with pregnancy outcome was observed (data not shown). Expression level of ovarian-steroid receptors in tumor cells must reflect the biological property of the endometrial epithelium where the tumor originated. Therefore, it is reasonable to presume that the abundance of ER and PgR in endometrial glands will provide a receptive microenvironment for embryo implantation through sufficient response to ovarian steroids.

In summary, by immunohistochemical analysis of MPA-treated patients' specimens, we demonstrate here that anti-tumor effect of MPA may be mediated by dephosphorylation of Akt, and that immunohistochemical analysis of phospho-Akt

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and PTEN may be able to predict the outcome of conservative MPA therapy in CAH and IaG1. It is also shown that ER and PgR expressions may correlate with pregnancy outcome of infertility treatment following MPA therapy. Although further accumulation of cases is required to confirm our proposal, the current observations provide novel implications for the treatment selection in young patients with CAH or IaG1 who are willing to preserve their reproductive potential. Additionally, our data suggest that newly discovered Akt inhibitors may be possible chemotherapeutic agents for Akt-activated endometrial tumors [31,32].

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Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1

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Background: Use of antiretroviral drugs has reduced the mortality rate for HIV infection and many HIV-discordant couples wish to have children. It is possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen.

Methods: An improved swim-up method was used to collect HIV-free spermatozoa from the semen of HIV-positive males. Diluted semen was layered over a Percoll solution with a continuous density gradient of 30–98%, and then centrifuged. The bottom layer was collected by cutting the end from the tube and the sperm suspension was collected using the swim-up method. Spermatozoa were tested by nested polymerase chain reaction (PCR) for HIV-1 RNA and DNA, with a detection limit of one copy. Spermatozoa were used for assisted reproduction in 43 couples.

Results: HIV-1 RNA and proviral DNA were not detected by nested-PCR assay in all 73 of the collected spermatozoa samples from 52 patients. The HIV-1-negative sperm was used for *in vitro* fertilization in 12 couples and for intracytoplasmic sperm injection in 31 couples. No detection of HIV-1 RNA or proviral DNA in the culture medium of the fertilized eggs was confirmed again before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Conclusions: HIV-negative spermatozoa could be obtained from semen of HIV-positive men. The method involves no risk of HIV transmission to female partners and their children. © 2006 Lippincott Williams & Wilkins

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Introduction

Since the mid-1990s, the use of HAART has spread, and the AIDS mortality rate has decreased by more than 80% in the industrialized world [1]. HIV infection/AIDS is becoming a controllable chronic infection and HIV-infected individuals are now living longer. Many HIV-positive people are getting married and wishing to have children.

Semprini *et al.* [2] reported that they had conducted artificial inseminations in more than 2000 HIV-discordant couples (HIV-positive male and HIV-negative female) using their swim-up method, and that no HIV transmission was observed. However, their method may be suboptimal because it has not been proven to remove HIV RNA completely, and they did not measure proviral DNA in infected cells in the semen. Zhang *et al.* [3] reported that HIV may be present as proviral DNA in seminal cells in HIV-infected men who have achieved undetectable levels of viral RNA in plasma with HAART, and this HIV could be capable of sexual transmission. It has not been determined whether HIV is attached to spermatozoa or whether spermatozoa can be infected with HIV [4,5]. Therefore, contraception is recommended for HIV-discordant couples, even if HIV RNA is undetectable in plasma [3].

Authorities in different countries have different opinions concerning the use of assisted reproductive technology using spermatozoa collected by the swim-up method [6–8]. However, it would be possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen. This study examines an improved swim-up method for isolating HIV-free sperm and its use in assisted reproductive methods.

Methods

This clinical study was approved by the ethics committees of Niigata University, Ogikubo Hospital, Keio University and Kyorin University. All of the couples visited the Hematology Department of Ogikubo Hospital and received counselling and explanations of the clinical study. Informed consent was obtained from all participating couples. Semen was obtained by masturbation, and then tested for sperm concentration, motility and deformity.

Percoll preparation

An isotonic solution of Percoll (Amersham Life Science, Tokyo, Japan) was made by dissolving 980 g Percoll in 10.0 ml 2.0 mol/l Hepes-NaOH, pH 7.4, 10.0 ml human serum albumin (25%w/v), 0.05 g fosfomycin and 0.05 g cefarotin. The resulting 98% Percoll solution was sterilized with a Millipore filter (0.45 μm pore size).

Semen pretreatments

The procedure is shown in Fig. 1. Ejaculates were diluted twice with Hanks solution, followed by standing in a test tube for 10 min to precipitate filterable micro-calculus, then filtered through an ART filter (20 μm clearance; ART filter, Nipro, Osaka, Japan) to remove fibers, micro-calculus and mucinous debris. The upper phase of sperm suspension was loaded onto 6 ml Percoll linear gradient from 98% to 30% in a separable fine-neck tube (Nipro) and centrifuged at 400 × *g* for 30 min. The separable fine-neck tube was made of glass, and its bottom was squeezed to minimize the volume of sediment. To recover the sperm precipitated in the bottom tip, the top of the tube was plugged with a rubber cap, and the middle of the squeezed bottom was snapped off with an ampoule cutter.

Motile sperm were separated by the modified swim-up method. A fine glass capillary was inserted in 2 ml of the medium in a vial, then a needle tip was introduced to the bottom through the inner capillary. The motile sperm were allowed to swim up at 37°C in an incubator with 5% CO_2–air. After 60 min, 1 ml of upper layer was collected, containing the sperm that had swum up.

The sperm suspension was divided into two portions. One was used for HIV assessment, and the other was cryopreserved with KS-II medium [9] in a liquid nitrogen container.

Standard HIV-1 materials

MOLT-4 cells infected with HIV_{LAI} and its culture supernatant were used as standards for HIV-1-infected cells and virus stock, respectively. RNA purified from virus stock and the pNL4-3 plasmid [10] were the standards for HIV-1 RNA and DNA, respectively. The concentrations of the standard HIV-1 DNA and RNA were determined by spectrophotometry and the null-class equation of the Poisson distribution of the reverse transcriptase (RT)-nested polymerase chain reaction (PCR). Cells were counted using a Burker–Turk hemocytometer (Emergo, Landsmeer, the Netherlands). The virion concentration was considered to be half the virus RNA concentration.

Detection of HIV-1 RNA and DNA

The samples of sperm suspension, culture medium or plasma were centrifuged at 35 500 × *g* for 1 h at 4°C. RNA and DNA were extracted from the precipitate using QIAamp UltraSens Virus Kit (Qiagen, Tokyo, Japan). One fourth of the eluate was tested in quadruplicate by RT-nested PCR as follows. The RT reaction was performed by incubation at 42°C for 10 min in a

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20 μl solution consisting of 1× PCR buffer II (10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl; Perkin Elmer Life Sciences, Yokohama, Japan), 3 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 0.1 μmol/l primer GA1R (5′-CCCAGGATTATCCATCTTTTATAG-3′, 1595–1572 [10]), 4 U RNasin (Promega, Tokyo) and 20 U SuperScript II (Invitrogen, Tokyo, Japan). The whole RT product was subjected to a first-round PCR in a 50 μl solution consisting of 1× PCR buffer II, 4 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 0.2 μmol/l primers GA1F (5′-TGTTAAAAGAGACCATCAATGAGG-3′, 1388–1411) and GA1R and 0.5 U AmpliTaq (Perkin-Elmer). Then, 1 μl of the first-round PCR product was used in the second-round PCR in a 50 μl solution containing primers GA2F (5′-GGCCAGATGAGAGAACCAAGG-3′, 1465–1485) and GA2R (5′-CATCCTATTTGTTCCTGAAGGGTAC-3′, 1535–1511) and the other components in first-round PCR. The primers were located in *gag* p24. The thermal profile of PCR in GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan) was 94°C for 2 min; three cycles of 94°C for 5 s, 48°C for 10 s and 72°C for 15 s; 22 cycles of 94°C for 5 s, 60°C for 10 s and 72°C for 15 s; with a final cycle of 72°C for 1 min and then the mixture kept at 4°C. The PCR products were electrophoresed through a 2.0% agarose gel in the presence of 0.5 μg/ml ethidium bromide and photographed under ultraviolet illumination. Throughout the procedure, the medium used for washed sperm or fertilized eggs was the negative control and this medium with 10 virions added was the positive control. The whole process took approximately 5 h. For samples of peripheral blood mononuclear cells (PBMC), DNA was extracted using QIAamp DNA Kit (Qiagen) and 0.5 μg of the DNA was tested in triplicate by the PCR procedures omitting reverse transcription. Competitive RT-nested PCR was performed as previously described [11].

Infectivity of HIV-1 during incubation

After incubation at 37°C under 5% CO_2 for various periods, the virus stock was added to 5×10^6 stimulated donor PBMC in 1 ml RPMI 1640 medium supplemented with 30% immobilized fetal calf serum and 70 U/ml human recombinant interleukin 2 (Shionogi, Osaka, Japan), and further incubated for 5 days. The culture supernatants were tested for p24 concentration with VIDAS HIV P24 II (BioMérieux, Tokyo, Japan).

Clinical study

If the HIV-1 testing for virion RNA and proviral DNA was negative, the other portion of frozen sperm was thawed for use in assisted reproduction. Mature eggs were obtained by means of ovulation-inducing drugs, and then placed in a dish containing 3 ml RPMI culture medium (20% albumin). The HIV-1–negative sperm solution was introduced to eggs by means of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), and the dish containing treated eggs was incubated at 37°C under 5% CO_2 for 48 h. Before embryo transfer, the culture medium for the fertilized egg was collected and tested for the presence of HIV-1 again. Only when HIV-1 RNA and DNA assays by nested PCR were negative was embryo transfer conducted. All the female partners who underwent assisted reproductive therapy, even those who did not conceive successfully, were tested for HIV antibodies, HIV-1 RNA and proviral DNA in the blood at 1 and 3 months after the assisted reproductive technique and after delivery. The babies were tested for HIV RNA and proviral DNA in umbilical cord blood at birth and in blood until 6 months after birth.

Results

Sensitivity of the HIV-1 RNA/DNA test

The procedure to detect a single copy of either HIV-1 virion RNA or proviral DNA in sperm suspensions (the HIV-1 RNA/DNA test) was developed by selecting and improving techniques in three main steps (collection of HIV-1 virions and infected cells by centrifugation, extraction of viral RNA and DNA with silica-gel-membrane technology, and the detection of the viral RNA and DNA by nested PCR) to achieve zero apparent loss in recovery at each step. First, the exact virion concentration of the standard HIV_{LAI} virus stock was determined by direct RT-nested PCR at endpoint dilution by using the null-class equation of the Poisson distribution. Then, one virion of HIV_{LAI}, on average, was added to 1 ml Sydney IVF medium (Cook, Tokyo, Japan) and the whole procedure was initiated. When one fourth of the eluate from an extraction column was examined (replicated four times) with RT-nested PCR, 12 of

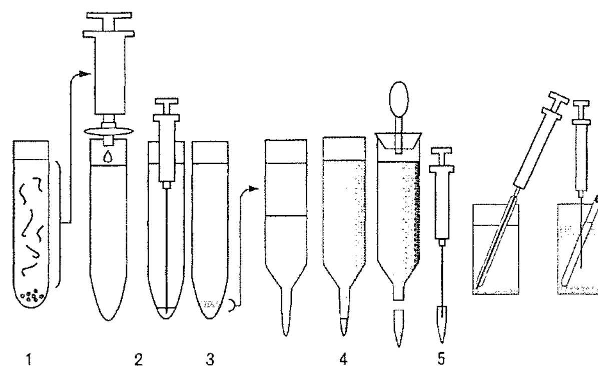

Fig. 1. Revised swim-up method to remove HIV completely. (1) The semen is diluted and debris allowed to precipitate. (2) The suspension is filtered, 0.1 ml Percoll added to the bottom, and the tube is centrifuged. (3)The sperm sediment is layered onto a linear gradient of Percoll (30–98%). (4) After centrifugation, the sediment is recovered by cutting the tube. (5) The sperm suspension is introduced into the bottom of the culture medium using a microtube. (6) The sperm that swim up are recovered.

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20 samples exhibited at least one band in four reactions (Fig. 2). Next, a single MOLT-4 cell chronically infected with HIV_{LAI} was added to 1 ml Sydney IVF medium and subjected to the HIV-1 RNA/DNA test without reverse transcription. RT-nested PCR showed that 6 of 10 samples exhibited positive reactions. The ratios of positive reactions for virions (60%) and infected cells (60%) were in close agreement with that predicted from the Poisson distribution (63%), providing evidence that the protocol has the ability to detect RNA/DNA in a single virion as well as in a single infected cell when present in as much as 1 ml of IVF medium. To study the influence of the presence of sperm in the medium on the sensitivity of the test, two sets of five samples containing 0.5, 1, 2, 4 and 8×10^6/ml spermatozoa in Sydney IVF medium were tested; one set was mixed with 50 virions and the other set with 100 infected cells. The numbers of virion RNA and proviral DNA from sperm-containing samples that were determined by competitive PCR varied in the range 75–112 copies (note two RNA copies/virion) and 96–122 copies, respectively, in a manner that was not dependent on the sperm quantity. These results strongly suggest that the protocol can detect a single virion or infected cell even in the presence of up to 8×10^6 spermatozoa per sample.

Removal of HIV-1 virions and infected cells from mixed semen by sperm-washing

To assess the efficiency of sperm-washing procedures with Percoll density gradient centrifugation and swim-up for removal of HIV-1 from semen, HIV-1 virions or HIV-1–infected cells were added to healthy donor semen. When 2×10^7 virions HIV-1 were mixed with 1.6 ml healthy donor semen containing 6.3×10^7 spermatozoa/ml, 63 copies of HIV-1 RNA were detected after centrifugation but no HIV-1 RNA was detected after swim-up. When 5×10^5 HIV-1–infected cells were mixed with 1.6 ml of healthy donor semen containing 6.3×10^7 spermatozoa/ml, no HIV-1 DNA was detected after either centrifugation or swim-up. The sperm suspension collected after swim-up was 1.0 ml in volume and contained 50 000 spermatozoa of 100% motility.

Decay of infectivity of HIV-1 during incubation

A virus solution of HIV_{LAI} was incubated in culture medium for various periods and the p24 production ability was quantified in stimulated PBMC to evaluate the stability of HIV-1 *in vitro* with regard to infectivity. Infectivity decreased semiexponentially with a half-life of approximately 13 h.

Results of the clinical study

A total of 52 HIV-1-positive individuals participated in the clinical study (Table 1); 29 were haemophiliacs and 23 had become infected through sexual contact. The median age was 33 years (range, 27–44) in the 16 untreated individuals, 34 years (range, 28–41) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 32 years (range, 20–51) in patients receiving HAART and with viral load < 50 copies/ml. Median plasma viral load was 17 500 copies/ml (range, 70–100 000) in the untreated group and 1500 copies/ml (range, 54–31 000) in patients receiving treatment and with a viral load $\geq$ 50 copies/ml.

Among 48 patients whose partner had assisted reproductive therapy, the median plasma viral load was 17 500 copies/ml (range, 70–100 000) in 15 patients in the untreated group, 4800 copies/ml (range, 54–31 000) in 10 patients receiving antiretroviral treatment and with viral load $\geq$ 50 copies/ml, and < 50 copies/ml in 23 patients taking HAART. Median CD4 cell count was 365 cells/μl (range, 66–1071) in the untreated group, 457 cells/μl (range, 60–652) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 399 cells/μl (range, 41–792) in patients receiving HAART and with viral load < 50 copies/ml. The median sperm count of the HIV-positive males was 47×10^6/ml (range, 0–82) in the untreated group, 41×10^6/ml (range, 0–65) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 35×10^6/ml (range, 0–120) in patients receiving HAART and with viral load < 50 copies/ml.

Azoospermia occurred in four patients, who were excluded from this study.

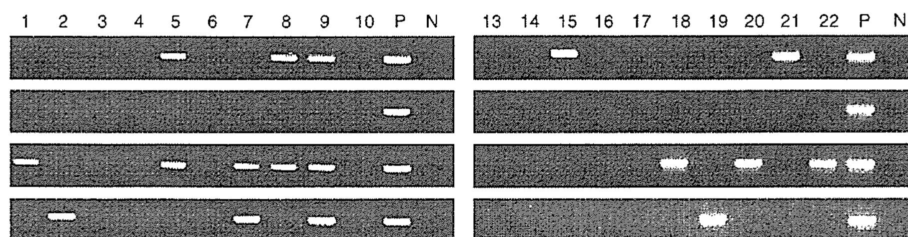

Fig. 2. Reverse transcriptase nested polymerase chain reaction (RT-nested PCR) capable of detecting a single copy of HIV-1 RNA. One virion of $HIV\text{-}1_{LAI}$, on average, was added to 20 sets of 1 ml Sydney IVF medium and then tested with the RT-nested PCR. When one fourth of the eluate from an extraction column was examined (in quadruplicate) with RT-nested PCR, 12 of 20 sets (lanes 1–10 and 13–22) exhibited at least one band in four reactions. Lanes P, positive control using 10 copies of $HIV\text{-}1_{LAI}$ RNA; lanes N, negative control with no HIV-1 RNA.

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Table 1. Characteristics of male patients with HIV infection.

	Untreated men ($n = 16$)	Men treated with HAART: Viral load ≥ 50 copies/ml ($n = 13$)	Men treated with HAART: Viral load < 50 copies/ml ($n = 23$)
Median age [years (range)]	33 (27–44)	34 (28–41)	32 (20–51)
Route of infection			
Haemophilia	10	8	11
Sexually transmitted	6	5	12
Median HIV viral load in serum [copies/ml (range)]	17 500 (70–100 000)	1500 (54–31 000)	< 50
CD4 cell count [cells/μl (range)]	365 (66–1071)	457 (60–652)	399 (41–792)
Sperm concentration [$\times 10^6$/ml (range)]	47 (0–82)	41 (0–65)	35 (0–120)
Azoospermia (No.)	1	2	1

In all patients, the median motility rate was 28%, and the median incidence of morphologically normal spermatozoa was 12%. The median concentration of spermatozoa in patients (excluding the four patients with azoospermia) was 42×10^6/ml (range, 3–120) and 52×10^6/ml (range, 0–170) spermatozoa were collected after the Percoll centrifugation. The median motility rate was 28% and 45% before and after the Percoll procedure. Following the swim-up method, there were 1.5×10^6/ml (range, 0–11) collected spermatozoa, and the motility rate was 100%. Spermatozoa could be collected by the swim-up method in 73 semen samples from the 48 patients. No HIV-1 RNA or proviral DNA was detected in any sperm suspensions collected after the swim-up procedure. The HIV-1-negative sperm was used for IVF in 12 couples and for ICSI in 31 couples. HIV-1 RNA or proviral DNA could not be deptected in the culture medium of the fertilized eggs before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Discussion

This study demonstrated that it is possible to detect a single copy of HIV-1 RNA or proviral DNA, and that HIV-negative spermatozoa can be obtained from the semen of HIV-positive males with the careful use of density gradient centrifugation and the swim-up technique. There has been no HIV-1 transmission in any of the female partners who underwent IVF or ICSI, nor in any of the babies.

Some studies have indicated that HIV can bind and enter into spermatozoa [4,5,12,13]. However, CD4 is not expressed on the surface of spermatocytes or spermatozoa [14,15]. Brogi *et al.* [4] have reported that HIV can attach to the surface glycoprotein of spermatozoa. In children at birth, the infection route is considered to be mother to child [16], and there is no case report of a child or embryo who has been infected with HIV via spermatozoa. It has also not been proven that a spermatid could be infected with HIV during spermatogenesis. This study showed that spermatozoa collected by the swim-up method were neither infected with HIV-1 nor had HIV-1 attached to them.

Semen contains spermatozoa, seminal plasma, white blood cells, microbes, metallic crystals and fibres of underwear. If components with higher density than spermatozoa are in a sample at centrifugation, those components may bring viruses and infected cells down to the bottom sperm fraction. Therefore, in our technique, we left diluted semen undisturbed to settle heavy components, and then took the sperm-containing upper fraction. If the sperm fraction (the bottom layer) following Percoll centrifugation is pipetted through the other denser layers, as is commonly done, HIV may contaminate the sperm fraction via the tube wall. In this study, we sealed the top of the tube after centrifugation and collected the sperm fraction by cutting off the bottom layer, which prevented contamination from the higher layers.

Gomibuchi *et al.* [17] reported that their method could not reduce HIV-1 RNA in semen to < 100 copies/ml in 55.6% of patients. Kuji *et al.* [18] have reported that the use of endotoxin-free Pureseption for semen processing had a lower elimination rate for HIV than the Percoll method. Some groups have used a swim-up technique in which the spermatozoa collected after centrifugation with a separating solution were washed with a culture medium and layered below the medium, followed by swim-up. Because the difference in the specific gravity of the sperm suspension and that of the culture medium is small, HIV and mononuclear cells may easily diffuse to the top layer during the swim-up method [17]. The actual procedures of the swim-up method, such as semen-washing techniques, the materials used in centrifugation, the concentrations of separating solutions, and the methods used to collect the bottom layer (sperm fraction) vary among researchers [17,19,20]. Therefore, it is

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considered that the HIV elimination rate will also vary. Our improved swim-up method provides a safer procedure for use in assisted reproductive techniques.

Semprini *et al.* [2] have reported that HIV transmission has not occurred in over 2000 patients who underwent artificial insemination using their method. Their successful results may be explained by the fact that infectious HIV is less than 1/10 000 of all HIV virions [11,21,22] and that removal of the HIV-producing mononuclear cells by the swim-up method is a major factor in reducing infection risk. We have reported that a female was infected with HIV-1 after six artificial insemination procedures using sperm prepared only by centrifugation in another hospital [23]. Artificial insemination should not be performed when inadequate HIV elimination methods are used or when the absence of HIV is not confirmed by highly sensitive tests.

Most HIV-infected patients in this study had low sperm counts and sperm motility rates, and provided a small number of spermatozoa after the swim-up method. As we try to achieve higher virus elimination rates, the number of collected spermatozoa becomes small. Ohl *et al.* [24] reported no pregnancies after artificial insemination using sperm obtained by the swim-up method. If it takes too long for PCR procedures, or if spermatozoa are frozen, the fertilization ability of the spermatozoa may be decreased and the probability of pregnancy may be low. It is difficult to confirm rapidly the removal of HIV-1 RNA and DNA in spermatozoa actually used for artificial insemination. CD4 and chemokine receptors are not expressed on eggs [25] and, therefore, eggs cannot become infected with HIV in the sperm suspensions collected using the swim-up method even if HIV is present in the suspension. If the suspensions are contaminated with a small amount of HIV, the infectivity of the HIV would still decrease to below 1/10 after a 2 day incubation. In addition, in IVF or ICSI, it is possible to confirm the absence of HIV-1 in the culture medium of fertilized eggs before embryo transfer. Therefore, we conducted IVF or ICSI using frozen spermatozoa that had been confirmed negative for HIV-1.

In conclusion, we have demonstrated that it is possible to collect spermatozoa with evidence of the absence of HIV-1 RNA and proviral DNA from semen of HIV-infected males. Whatever method is used for assisted reproductive technique and for removal of HIV from semen to reduce the risk of secondary transmission, it is essential to confirm the absence of HIV-1 RNA and proviral DNA in the sperm preparation used for the assisted reproductive technique with the most sensitive tests possible.

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Possible Involvement of CD81 in Acrosome Reaction of Sperm in Mice

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ABSTRACT Tetraspanin CD81 is closely homologous in amino acid sequence with CD9. CD9 is well known to be involved in sperm–egg fusion, and CD81 has also been reported to be involved in membrane fusion events. However, the function of CD81 as well as that of CD9 in membrane fusion remains unclear. Here, we report that disruption of the mouse CD81 gene led to a reduction in the fecundity of female mice, and CD81−/− eggs had impaired ability to fuse with sperm. Furthermore, we demonstrated that when CD81−/− eggs were incubated with sperm, some of the sperm that penetrated into the perivitelline space of CD81−/− eggs had not yet undergone the acrosome reaction, indicating that the impaired fusibility of CD81−/− eggs may be in part caused by failure of the acrosome reaction of sperm. In addition, we showed that CD81 was highly expressed in granulosa cells, somatic cells that surround oocytes. Our observations suggest that there is an interaction between sperm and CD81 on somatic cells surrounding eggs before the direct interaction of sperm and eggs. Our results may provide new clues for clarifying the cellular mechanism of the acrosome reaction, which is required for sperm–egg fusion. *Mol. Reprod. Dev.* © 2007 Wiley-Liss, Inc.

Key Words: CD9; acrosome reaction; fertilization; mice; zona pellucida

INTRODUCTION

Fertilization is accomplished by the direct interaction of sperm and eggs, a process mediated primarily by predicted, but yet unidentified gamete membrane proteins. In fertilization, the acrosome reaction is a change in sperm that is required for penetration into the zona pellucida, the egg coat, and facilitates the subsequent fusion with the egg plasma membrane (Moreno and Alvarado, 2006). Zona pellucida protein 3 (ZP3), one of the components forming the meshwork of the zona pellucida, has been considered to be the prime physiological inducer of the acrosome reaction in sperm, although the frequency of acrosome reaction is low after incubation with recombinant ZP3 (Beebe et al., 1992). This discrepancy suggests that, besides ZP3, unknown major factor(s) might be responsible for the acrosome reaction. To date, despite the importance of the acrosome reaction in fertilization, the underlying cellular mechanisms that regulate the acrosome reaction remain unclear.

Two tetraspanins, CD9 and CD81, are known to be important in the membrane fusion events in various biological systems. In virus-host cell fusion, human CD81 has been identified as a co-receptor for hepatitis C virus (Higginbottom et al., 2000; Cormier et al., 2004). Both CD9 and CD81 have been implicated in myoblast fusion (Tachibana and Hemler, 1999; Schwander et al., 2003) and monocyte/macrophage fusion in mice (Takeda et al., 2003). Recent studies using gene-targeting techniques demonstrated that female mice carrying a deletion of the CD9 gene produce eggs that mature normally but are defective in sperm–egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Takahashi et al., 2001). CD81 has also been reported to be expressed on the plasma membrane of unfertilized mouse eggs (Takahashi et al., 2001). Furthermore, CD81−/− mice have been reported to have defects in reproduction after several generations of backcrossing (Deng et al., 2000).

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Recently, Rubinstein et al. (2006) provided more detailed data showing that eggs of CD81−/− mice are unable to be fertilized with sperm, although the degree of the defect appeared not to be severe compared with that of CD9−/− eggs. Moreover, injection of CD9−/− eggs with mouse CD81 mRNA revealed that mouse CD81 was only moderately effective at reversing the infertility of CD9−/− eggs (Kaji et al., 2002). These findings taken together indicate that CD81 and CD9 each have different roles in fertilization.

Here we studied the role of CD81 in fertilization by in vitro fertilization (IVF) and immunohistochemical analysis, and propose a possible role of CD81 in the acrosome reaction in sperm.

MATERIALS AND METHODS

Animals

CD81−/− mice (Miyazaki et al., 1997) were kindly provided by Dr. Miyazaki and were backcrossed to C57BL/6 mice. Genotyping was carried out using polymerase chain reaction as previously described (Miyazaki et al., 1997). To visualize acrosome-intact sperm, EGFP-transgenic mice expressing EGFP in the acrosomes were generated by pronuclear injection of constructs carrying the EGFP gene driven by the mouse acrosin promoter (Nakanishi et al., 1999) and the DsRed2 gene tagged with a mitochondrial transport signal and driven by the CAG promoter into fertilized eggs of BDF1 mice (unpublished information). After the sperm were acrosome-reacted, EGFP was lost from the sperm heads and DsRed remained in the mitochondria of the mid-piece region. All animal procedures were performed according to protocols approved by the National Center for Child Health and Development and use committees.

Egg Collection

Female mice (aged 8–15 weeks) were injected with 5 U of hCG (Gonatropin; Aska Pharmaceutical Co., Ltd, Tokyo, Japan) 48 hr after administration of 5 U of PMSG (Serotropin; Aska Pharmaceutical Co., Ltd). Ovulated eggs were collected from the oviductal ampulla 13.5–15 hr after hCG injection, and placed in 100-µl drops of TYH medium equilibrated with 5% CO_2 in air at 37°C. Cumulus cells were removed with 300 IU/ml of hyaluronidase (H-3506, Sigma-Aldrich, Missouri, MO), and eggs were incubated with a defined number of sperm.

Sperm Preparation and In Vitro Fertilization

Sperm were collected by squeezing two cauda epididymides of 8- to 10-week-old B6C3F1 or transgenic male mice in a well containing 100-µl of TYH medium. Sperm were incubated at 37°C in 5% CO_2 for 90 min before being mixed with eggs derived from wild-type or CD81−/− female mice. The final concentration of sperm added to an egg-containing drop was 1.5×10^5 sperm/ml. To examine the rate of fertilization, we counted the number of eggs at the two-cell stage 24 hr after incubation with the sperm. For counting the number of fused sperm, the zona pellucida was removed from the eggs by a brief incubation in acid Tyrode solution, and sperm were incubated with eggs preloaded with 4′,6-diamidino-2-phenylindole (DAPI) for counting the number of sperm fused with eggs (Yamagata et al., 2002). For counting the number of acrosome-intact sperm, EGFP-expressing sperm were incubated with zona-intact CD81+/+ or CD81−/− eggs. The eggs were all subjected to confocal microscopic analysis for the presence of sperm exhibiting red and green fluorescence or red fluorescence alone within the perivitelline space 4 hr after incubation.

Immunostaining

For immunostaining of cryostat sections, ovaries from 8- to 10-week-old wild-type C57BL/6 females were fixed in 2% paraformaldehyde in PBS (−) for 2 days at 4°C, and then immersed in 30% sucrose in PBS (−) for more than 2 days at 4°C, embedded in Tissue-Tek OCT compound (Sakura Finetek Co., Tokyo, Japan), and finally frozen before serial cryostat sectioning (8 µm in thickness). Slides were fixed in an acetone and incubated with anti-CD81 antibody (Eat-1) diluted 1:300 in PBS (−) containing 0.1% bovine serum albumin (BSA), BSA/PBS (−), or anti-ZP3 antibody diluted 1:300 in BSA/PBS (−), overnight at 4°C. After washing three times with BSA/PBS (−), the samples were incubated with Alexa 546-conjugated goat anti-hamster IgG (A-21111, Invitrogen, California, CA) or Alexa 488-conjugated goat anti-rat IgG (A-11006, Invitrogen) for 2 hr at room temperature. After extensive washing, the slides were inspected for fluorescence using LSM 510 META confocal microscope.

Immunoblotting

Samples containing equal amounts of eggs were dissolved in nonreducing sample buffer and subjected to 12% SDS–PAGE according to procedures described previously (Miyado et al., 2000). After electrophoresis, the gels were transferred to PVDF membranes for immunoblot analysis. The blots were blocked in 1% nonfat dry milk, and were probed with the primary antibodies, anti-mouse CD81 antibody (Eat-1, BD Biosciences, California, CA) or anti-mouse CD9 antibody (KMC8, BD Biosciences). After washing in TBS-Tween buffer, the membranes were incubated with HRP-labeled secondary antibodies; goat anti-rat antibody or goat anti-hamster antibody. The expression level of immunoreacted products was determined by treatment of the blots with an ECL or ECL Plus Detection Kit (GE Healthcare Bio-Science Co., New Jersey, UK) and exposure to X-ray film at room temperature.

Statistical Analysis

Data from different groups were tested by the *t*-test for the significance of the difference between the means of two independent samples using the computer software KaleidaGraph (version 3.6, Synergy Software, Inc., Pennsylvania, PA).

RESULTS

Female Fertility Impaired by CD81 Deficiency

Figure 1A depicts the average litter size in matings of three genotypes of females, wild-type, CD81+/− and CD81−/− mice, with CD81+/− males over a period of 6 months. Although these females displayed normal mating behavior with the males (data not shown), the average litter size of CD81−/− females was markedly reduced relative to those of CD81+/− and wild-type mice (on average, 1.3 ± 2.5 vs. 11.3 ± 1.3 and 11.0 ± 0.8) (Fig. 1A). To examine the oocyte maturation and ovulation, we also collected the eggs from mice superovulated by stimulation with exogenous gonadotropin. The eggs collected from CD81−/− mice were indistinguishable with regard to morphology and number (on average, 18.0 ± 2.8) from those wild-type and CD81+/− mice (on average, 19.9 ± 1.7 and 21.7 ± 2.8) (Fig. 1B). Therefore, the reduction in fertility of CD81−/− females did not seem to be due to defects of ovulation or oocyte maturation.

Involvement of CD81 in Fertilization

To clarify the cause of the reduced fertility of CD81−/− females, the function of CD81−/− eggs was further examined by IVF. When cumulus oocyte complexes (COCs) collected from CD81−/− or wild-type control mice were incubated with the wild-type sperm, the sperm could disperse cumulus cells, somatic cells surrounding eggs, and reach and apparently penetrate the zona pellucida of CD81−/− and wild-type eggs. However, the average rate of eggs developing to the two-cell stage was substantially decreased for CD81−/− eggs (on average, $15.0 \pm 2.5\%$) compared with that for wild-type eggs (on average, $65.0 \pm 10.8\%$) 24 hr after incubation with the sperm (Fig. 1D). Furthermore, in CD81−/− eggs, several sperm were observed in the perivitelline space (Fig. 1C). The delayed formation of two-cell embryos and the accumulation of more than one sperm within the perivitelline space in CD81−/− eggs demonstrate that CD81−/− eggs have impaired ability of fertilization. Subsequently, to examine the cause of the impaired fertilization, we performed IVF for CD81−/− eggs and wild-type eggs after the zona pellucida was removed using acid Tyrode solution (Fig. 2A,B). To measure the number of sperm fused with eggs, both types of eggs were preloaded with DAPI before incubation with wild-type sperm (Yamagata et al., 2002). One hour after insemination, estimation of the average number of sperm fused with one egg by measurement of DAPI fluorescence revealed that CD81−/− eggs showed a decreased number of fused sperm (on average, 1.21 ± 0.23) in comparison with the wild-type eggs (on average, 1.95 ± 0.27). Those results suggest that CD81 is involved in sperm–egg fusion, either directly or indirectly.

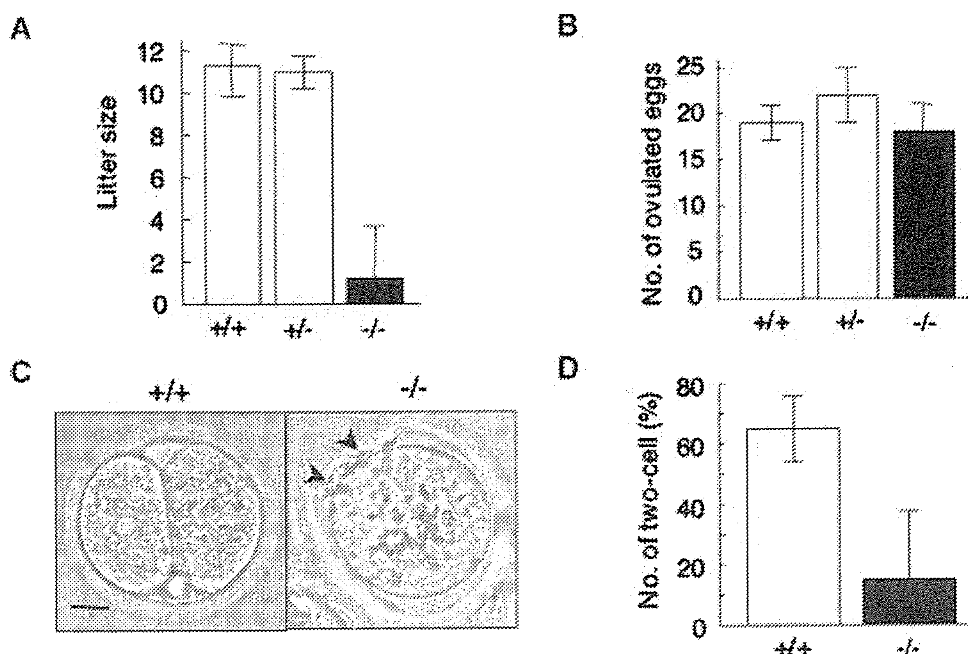

Fig. 1. Female infertility caused by CD81 deficiency. **A**: Average litter sizes of crosses between CD81+/− males and three types of females, wild-type, CD81+/− and CD81−/− mice. Each of the mating pairs was kept in a separate cage, and births over a 6-month period were monitored. Data of births during successive 2-month periods were grouped together, and the average litter size of wild-type, CD81+/− and CD81−/− females was calculated from data recorded for five mating pairs 8–15 weeks of age at the start of the experiment. **B**: Average number of ovulated eggs from wild-type, CD81+/− and CD81−/− female mice. The eggs were collected 13.5–16 hr after hCG treatment, and counted. **C**: Representative micrographs of CD81+/+ and CD81−/− eggs. The eggs were obtained 24 hr after incubation with the wild-type sperm. **D**: Average number of eggs that developed to the two-cell stage 24 hr after incubation with the wild-type sperm. The black bars show the results for CD81−/− eggs (A,B,D). Error bars represent SEM (A,B,D). Scale bar, 20 μm (C).

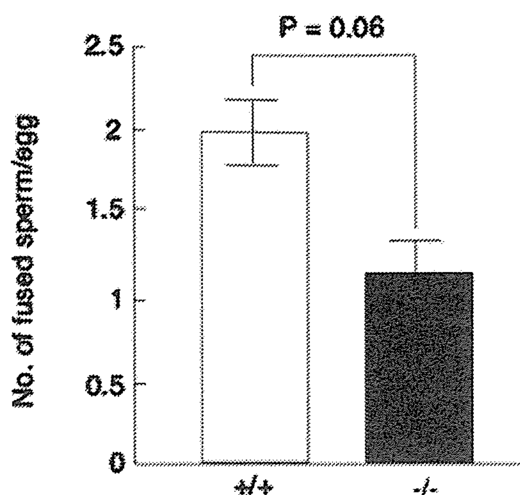

Fig. 2. In vitro sperm–egg fusion. Average number of sperm fused with wild-type or CD81−/− eggs after 3 hr of incubation. Error bars represent SEM. Data from different groups were tested by the *t*-test for the significance of the difference between the means of two independent samples ($P = 0.06$).

Expression of CD9 in CD81−/− Eggs

The mechanisms by which CD81 acts are still unclear. However, CD81 tends to form multimolecular complexes in which tetraspanins associate with specific proteins depending on the type of cell. In B cells, CD81 directly associates with CD19, taking part in the CD19-CD21-CD81 signaling complex (Pileri et al., 1998), which accords with the evidence that the expression of CD19 in bone marrow, spleen, and peripheral B cells is reduced in CD81−/− mice (Miyazaki et al., 1997). As previously mentioned, CD9 on the egg plasma membrane is required for fusion with sperm, and the impaired fusibility of CD81−/− eggs with sperm may likely be dependent on the expression of CD9. To investigate whether CD81 deficiency may cause downregulation of CD9 expression, the expression level of CD9 was examined (Fig. 3). We collected three types of eggs,

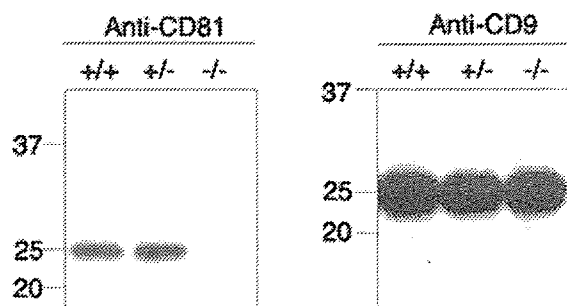

Fig. 3. The expression of CD9 in wild-type, CD81+/− and CD81−/− eggs. Proteins were isolated from the types of eggs indicated and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions. The proteins were electrophoretically transferred to a membrane, where they were probed with antibodies to CD81 (panel A) and CD9 (panel B). The proteins corresponding to each 110 egg (panel A) and 10 eggs (panel B) were analyzed.

wild-type, CD81+/− and CD81−/− eggs, 13.5–15 hr after hCG injection into mice, and examined the expression level of CD9 in comparison with that of CD81 by SDS–PAGE. The amounts of CD81 were invariable in wild-type and CD81+/− eggs, but CD81 was lost in CD81−/− eggs. By contrast, there were no significant differences in the expression of CD9 among these eggs. Therefore, the impairment of fertilization caused by CD81 deficiency cannot be attributed to decreased expression of CD9 in eggs.

Expression of CD81 During Folliculogenesis

The expression and localization of CD81 in ovarian tissues were immunohistochemically assessed using cryostat sections of adult wild-type ovaries. The follicles consist of immature eggs and granulosa cells that surround the egg; a single follicle usually grows to the preovulatory stage and releases the egg for potential fertilization (Buccione et al., 1990). Immunohistochemical staining with anti-CD81 mAb demonstrated that CD81 was continuously expressed in the egg and surrounding follicles (Fig. 4), and in cumulus cells surrounding ovulated eggs (data not shown). These data indicate that the sperm may encounter CD81 on the somatic cells surrounding eggs before direct interaction of sperm and eggs.

Possible Involvement of CD81 in Acrosome Reaction

Based on the localization of CD81 and the impaired fertilization of CD81−/− eggs, we speculated that the inability of wild-type sperm to fuse CD81−/− eggs might be due to impairment of prefusional stages, including the acrosome reaction. To examine the involvement of CD81 in the acrosome reaction of the sperm, CD81−/− eggs or wild-type eggs were incubated with the sperm collected from transgenic mice specifically expressing enhanced green fluorescent protein (EGFP) in the acrosomes (Fig. 5). The acrosome corresponds functionally to a lysosome and thus contains lysosomal enzymes (Moreno and Alvarado, 2006), and acrosin is a sperm acrosomal serine proteinase that is lost from the sperm head after the acrosome reaction is completed (Baba et al., 1994). Therefore, sperm expressing EGFP at the acrosomes in the heads are useful for the detection of acrosome-intact sperm. After 3 hr of incubation, we counted the number of acrosome-intact sperm within the perivitelline spaces. To count the sperm that had penetrated into the zona pellucida, the eggs were incubated with 3.0×10^5 sperm/ml. When the number of sperm within the perivitelline space were counted 3 hr after incubation with the eggs, we observed that an increased percentage ($8.5 \pm 2.3\%$) of the sperm that had penetrated into the perivitelline space of CD81−/− eggs exhibited EGFP fluorescence in their head portion. In contrast, very few sperm that had penetrated into the perivitelline space of wild-type eggs exhibited green fluorescence ($1.4 \pm 1.0\%$). These results suggest that the sperm that penetrated into the zona pellucida of the CD81−/− eggs were impaired in the acrosome reaction.

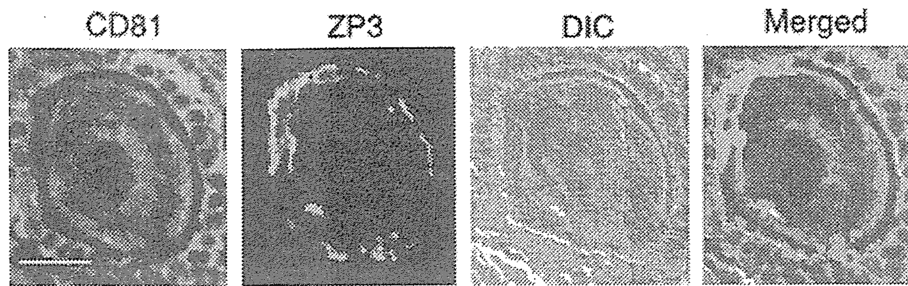

Fig. 4. CD81 is expressed at high levels in granulosa cells during oogenesis. Frozen sections of ovaries from wild-type mice were stained with anti-mouse CD81 mAb and with anti-ZP3 mAb. DIC represents a photograph taken by differential interference contrast. Scale bar, 20 μm. [See color version online at www.interscience.wiley.com.]

DISCUSSION

CD81 has been suggested to be a protein playing a role in membrane fusion events, but the function of CD81 in sperm–egg fusion remains unknown. As suggested by Rubinstein et al. (2006), CD9 and CD81 may have different roles in sperm–egg fusion. This notion is supported by the following facts: (1) deletion of a single gene, CD9 or CD81, causes impaired fertilization, and the expression of CD9 on eggs is not perturbed by CD81 deficiency, and (2) CD9−/− eggs injected with mRNA encoding CD81 cannot be fully rescued to the same degree as those injected with CD9 mRNA (Kaji et al., 2002).

Generally, the acrosome reaction is a change in the membrane of sperm that are activated for penetration into zona pellucida and facilitates the subsequent fusion with the egg membrane (Baba et al., 1994). During the acrosome reaction, the disruption of the acrosome covering the sperm head causes the release of acrosin and other proteolytic substances. As previously reported (Moreno and Alvarado, 2006), these materials included in the acrosome are important for the penetration of sperm into the zona pellucida and for sperm–egg fusion, but the molecular mechanism underlying the acrosome reaction is largely unknown. When wild-type eggs were incubated with sperm expressing EGFP in the acrosomes, we found the presence of acrosome-intact

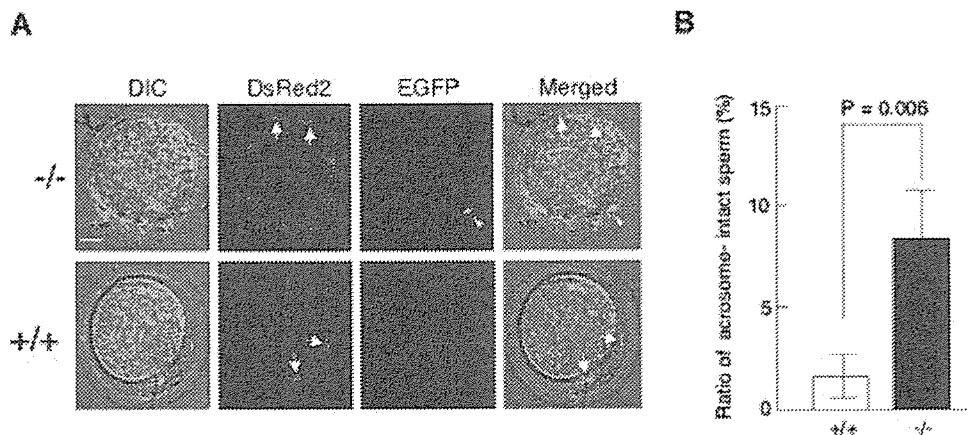

Fig. 5. In vitro fertilization assay for acrosome reaction. A: Representative photographs. CD81−/− eggs were incubated with transgenic sperm expressing EGFP at acrosomes in the sperm heads. Eggs from wild-type females were also subjected to fertilization using the AR-GFP transgenic sperm as controls. Four hours after insemination, the eggs were inspected for fluorescence using a confocal microscope. As shown in the upper panel, some CD81−/− eggs had sperm with green fluorescence (indicated by arrowheads) in their head region in the perivitelline space, while almost no wild-type eggs had such types of sperm (lower panel). Photomicrographs taken under light (DIC); photomicrographs taken for detecting DsRed2 translocated to mitochondria by the retention signal (Mt-DsRed2) and specifically expressed in the mid-piece of sperm (indicated by arrows); photomicrographs taken for detecting EGFP-derived green fluorescence specifically expressed in the head region of sperm (indicated by arrowheads); merged images. Scale bar, 20 μm. B: Examination of acrosome reaction using EGFP-expressing sperm. CD81−/− or wild-type eggs were fertilized in vitro with epididymal sperm expressing EGFP in the acrosomes. Four hours after insemination, the sperm entering into the perivitelline space were inspected for fluorescence using a confocal microscope. Note that the number of sperm carrying intact acrosomes (exhibiting green fluorescence in the sperm head region, as shown in A) and entering into the perivitelline space of CD81−/− eggs was significantly higher than that of acrosome-intact sperm entering into the perivitelline space of wild-type eggs. Acrosome-intact sperm can easily be detected since they exhibit bright green fluorescence in their head region. The total number of sperm entered into perivitelline space can be counted by inspection for red fluorescence in the mid-piece of the sperm. [See color version online at www.interscience.wiley.com.]

sperm in the outer layer of the zona pellucida (data not shown), but almost all sperm that penetrated into the perivitelline space had lost the acrosome caps (Fig. 5). These findings suggest that the acrosome reaction may occur in the perivitelline space and/or inner layer of the zona pellucida.

Another possible reason for the failure of the acrosome reaction of EGFP-expressing sperm in CD81−/− eggs is that "zona hardening" in CD81−/− eggs may not be sufficient compared to that in wild-type eggs. The weakened zona hardening might permit the penetration of some acrosome-intact sperm into CD81−/− eggs. However, since proteins other than components forming the zona pellucida may be triggers for preventing polyspermy and zona hardening (Sun, 2003), it would be of interest to test whether CD81 and ZP3 interact with each other.

In conclusion, the results of our IVF experiments suggest the possible involvement of CD81 in the acrosome reaction of zona pellucida-penetrated sperm prior to the fusion of sperm with eggs. Extensive attempts to elucidate the role of CD81 in the acrosome reaction are now underway.

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