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

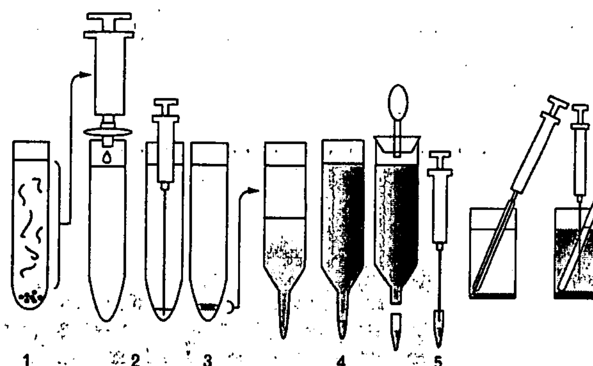

Fig. 1. Revised swim-up method to remove HIV completely. (1) The semen is diluted and debris allowed to precipitate. (2) The suspension is filtered, 0.1 ml Percoll added to the bottom, and the tube is centrifuged. (3)The sperm sediment is layered onto a linear gradient of Percoll (30–98%). (4) After 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 ≥ 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 ≥ 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 ≥ 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 ≥ 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 ≥ 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.

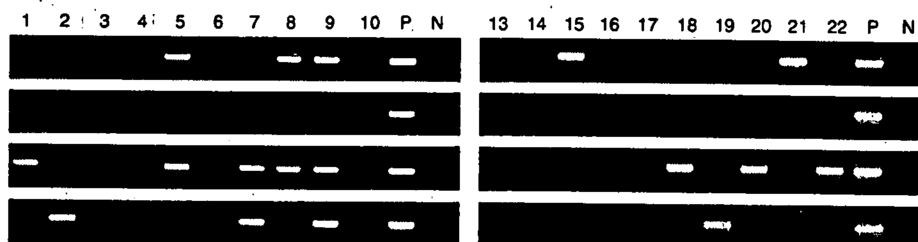

Fig. 2. Reverse transcriptase nested polymerase chain reaction (RT-nested PCR) capable of detecting a single copy of HIV-1 RNA. One virion of $HIV\text{-}1_{LAI}$, on average, was added to 20 sets of 1 ml Sydney IVF medium and then tested with the RT-nested PCR. When one fourth of the eluate from an 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 [× 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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Maintenance of In Vitro Granulosa Cell Function by Adenoviral Mediated Follicle Stimulating Hormone Receptor Gene Transduction

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Purpose: To maintain in vitro granulosa cell function by adenoviral-mediated FSHR gene transduction.
Methods: Rat granulosa cells were cultured and transduced with adenovirus carrying FSHR gene. The number of receptors and the rate of steroidogenesis were assessed.
Results: The number of FSHR on the granulosa cells was 4,874 per cell immediately after extraction, it was 2,176 by the third day, and had further reduced to 693 by the seventh day. On the third day of culture, the amount of production of estradiol by FSH stimulation also decreased to about one-quarter of the first day's quantity. Compared to the untransduced granulose cells, when the cells contained FSHR gene, the FSHR expression and steroidogenesis were both enhanced (2,176 vs. 7,206 per cell ($p < 0.001$) and 192 vs. 5940 pg/mL ($p < 0.01$), respectively).
Conclusion: Granulosa cell functions can be maintained or increased by novel gene therapy. This can be a useful component of assisted reproductive therapy.

KEY WORDS: Adenoviral vector; FSH receptor; granulosa cell; in vitro maturation.

INTRODUCTION

In vitro maturation (IVM) of cumulus–oocyte complexes (COC) potentially offers many advantages in assisted reproductive technology, as it would reduce the cost of treatment; decrease the risk of ovarian hyperstimulation syndrome; and increase the number of matured oocytes for fertilization.

To date, several successful births of normal babies have been achieved by in vitro matured human oocytes (1), although the implantation rate of embryos derived from in vitro matured oocytes remains low when compared with in vivo matured oocytes (2).

In mammalian species, oocytes retrieved from the antral follicles are capable of undergoing spontaneous nuclear maturation, but in general fail to complete cytoplasmic maturation (3). Establishment of the optimal culture conditions to induce synchronized nuclear and cytoplasmic maturation of oocytes is required.

Granulosa cells within the developing follicle play a crucial role in providing a suitable microenvironment for the maturation of oocytes; therefore coculture with granulosa cells may improve the developmental competence of in vitro maturing oocytes. Unfortunately, granulosa cells rapidly lose FSH receptor and steroidogenesis capability in tissue culture conditions (4).

In this study, we introduced the follicle stimulating hormone receptor (FSHR) gene into primary

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rat granulosa cells by replication-deficient adenoviral vector to maintain their response to FSH.

MATERIALS AND METHODS

Preparation of Culture Medium and Cell Culture

The primary culture of rat granulosa cells was done with Dulbecco's modified Eagle medium (DMEM, Immuno-Biological Laboratories, Fujioka, Japan) supplemented with 0.01% bovine serum albumin (BSA, Sigma, St. Louis, MO). Rat granulosa cells were cultured on plates coated with 20 ug/mL E-C-L Cell Attachment Matrix (Upstate biotechnology, Lake Placid, NY). To control for the influence of FSH and estrogen production, cells from COLO320 human colon adenocarcinoma cell line were used (5). These cells do not express the FSH receptor. They were cultured with DMEM supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS).

Both of the cells were cultured in a humidified atmosphere containing 5% carbon dioxide (CO_2) and 95% air at 37°C.

Laboratory Animals

Immature female Wistar rats (21 days old) were purchased from Charles River, Japan (Yokohama, Japan) and maintained under pathogen-free environment. These animals were given an injection of 2 mg diethylstilbestrol (Sigma) in 0.1 mL sesame oil (Kanto Chemical, Tokyo, Japan) once daily for 4 days to stimulate the development of multiple immature follicles. Two days later, the animals were sacrificed by CO_2 inhalation (6), followed by cervical dislocation. The ovaries were then rapidly removed for granulosa cell isolation.

Granulosa Cell Isolation and Culture

Granulosa cells were released by puncturing surface follicles on the ovaries with a hypodermic needle. The cell number was counted with a hemocytometer under a phase-contrast microscope and viability was assessed using trypan blue dye exclusion. The granulosa cells were then cultured in DMEM on E-C-L Cell Attachment Matrix coated plates in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C (7).

Receptor Binding Assay

Rat granulosa cells were cultured on Multiscreen 96-well filtration plates and (Millipore, Bedford, MA) coated with E-C-L. Each well contained 10^5 cells in 0.1 mL DMEM with 0.01% BSA. After an appropriate incubation period, FSH binding sites were measured by Radio Receptor Assay (RRA) on whole cultured cells using ^{125}I-FSH, with a specific activity of 2732–5529 Ci/mmol, purchased from NEN Life Science Products (Boston, MA).

The granulosa cells were incubated for 1 h at 37°C in 100 uL assay buffer, which consisted of 0.1% BSA and 0.1% sodium azide in phosphate buffered saline (pH 7.4), with an increasing amount of ^{125}I-FSH. Fertinorm P (Serono Laboratories, Aubonne, Switzerland) was added as unlabeled FSH to determine nonspecific binding. The incubation was terminated by the addition of 150 uL of ice-cold assay buffer. The unbound^{125}I-FSH was separated from bound^{125}I-FSH by centrifuging the filtration plates at 2000 rpm. Then the cells were successively and rapidly washed three times with 200 uL of ice-cold assay buffer. The filters were punched out and the amount of radioactivity was measured with the Auto Well Gamma System ARC-2000 (Aloka Co., Ltd., Mitaka, Tokyo).

Total binding was defined as the binding of ^{125}I-FSH without unlabeled FSH, and nonspecific binding was defined as the binding of ^{125}I-FSH, which was nondisplaceable with 1000-fold excess of unlabeled FSH. The specific binding was defined as the difference between the total binding of ^{125}I-FSH and the nonspecific binding. Equilibrium saturation curves were determined by incubation with increasing amounts of ^{125}I-FSH (200–1600 pM). All samples were assayed in triplicate wells and the results were processed in a Scat chard plot. The parameters Kd and B_{max} were determined for each experiment by fitting the data to the single-site binding equation ($B = B_{max} \times L/(Kd + L)$), where B is specific binding at free^{125}I-FSH concentration (L) using nonlinear least-square regression method).

Assays

Concentrations of estradiol in the culture medium were measured by radioimmunoassay using DPC estradiol kit (Diagnostic Products Corporation, Los Angeles, CA) in the presence of 4-androstene-3,17-dione (Sigma) as the substrate. Concentrations of cAMP were measured by radioimmunoassay using

the YAMASA RIA kit (Yamasa Shoyu, Choshi, Japan).

Construction of hFSHR Recombinant Adenovirus

The cDNA encoding the human FSHR (2393 bp) was kindly provided by Dr. Takashi Minegishi (Gunma University, Gunma) (8). Replication-defective adenovirus vector containing FSHR cDNA (AxCAFSHR) under CAG promoter was used for this study. AxCAFSHR was constructed by the COS-TPC method using an Adenovirus Expression Vector Kit (Takara Shuzo, Otsu, Japan) according to the manufacturer's instruction (9). The infectivity of recombinant adenoviruses was measured by an end-point cytopathic effect assay, and the 50% tissue culture infection dose (TCID50) was calculated (10). In our series, TCID50 approximately corresponds to plaque forming units (pfu). The titer of AxCAFSHR after final propagation was 4.5×10^9 pfu/mL. These studies were permitted by Niigata University recombinant DNA experiments safety committee.

Adenovirus Infection

Granulosa cells and COLO320 cells were seeded into 96-well (10^5 cells per well) tissue culture plates and allowed to attach overnight. The next day, medium and unattached cells were removed and the remaining cells were exposed to adenoviruses at MOI 100 (COLO320 cells) or MOI 10 (granulosa cells) in 40 uL of medium for 1 h at 37°C with occasional rocking. A total of 160 uL of fresh medium was added to each well and the wells were appropriately incubated.

Preparation of Probes

Probe for FSHR was generated by polymerase chain reaction (PCR) using murine genomic DNA as a template. The sequence of forward primer was 5′-GCAGGCTGATGCTGCTGGC-3′ and the sequence of reverse primer was 5′-GGCACCTTGAGGGAGGCAGAAAT-3′. The PCR product (418 bp) was separated by electrophoresis on 1% agarose gels, and extracted from the gel using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The purified products were labeled with ^{32}P using the Rediprime II DNA Labeling System (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA probe of beta-actin was labeled in the same way.

RNA Blot Analysis

Total RNA was extracted from COLO320 cells or granulosa cells by the guanidinium acid–thiocyanate–phenol chloroform method (11) using ISOGEN (NIPPON GENE, Tokyo, Japan). Ten micrograms of total RNA was separated by electrophoresis on denaturing agarose gels for the Northern blot analysis. This was subsequently transferred onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Northern blots were hybridized overnight at 45°C with the ^{32}P-labeled FSHR or beta-actin probe. The membrane was rinsed three times in 3 × SSC and exposed to Kodak BioMax MS film using intensifying screens, TranScreen HE (Eastman Kodak, Rochester, NY).

RESULTS

In Vitro FSHR Expression and Estradiol Production of the Granulosa Cells

Rat granulosa cells immediately after extraction expressed 4874.2 ± 1753.8 / cell FSHR.The number of receptors decreased as time passed (2176.0 ± 1288.8, 827.9 ± 351.7, and 692.6 ± 188.8 on the third, fifth, and seventh day of culture, respectively) without any addition of hormones (Fig. 1).

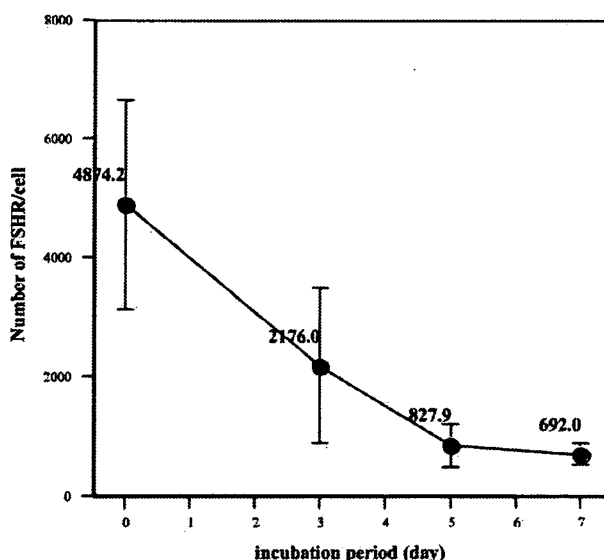

Fig. 1. The number of FHSR expressed on rat granulosa cells on days 0, 3, 5, and 7 of culture. Data shown are the mean of three experiments.

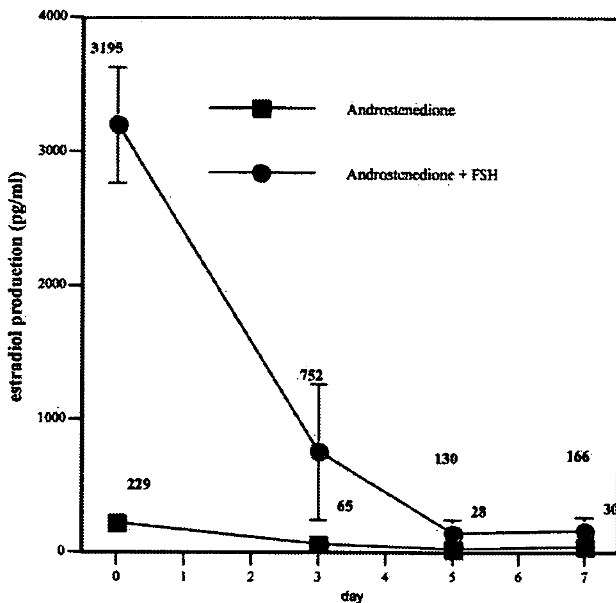

Fig. 2. The amount of estradiol production of rat granulosa cells on days 0, 3, 5, and 7 of culture. After washing plate, 50 ng/mL of androstenedione (■), which was a substrate for aromatase, or androstenedione plus 100 mIU/mL of FSH (●) were added to the culture medium. The medium was collected 48 h after the addition of hormones for estradiol assay. Data shown are the mean of three experiments.

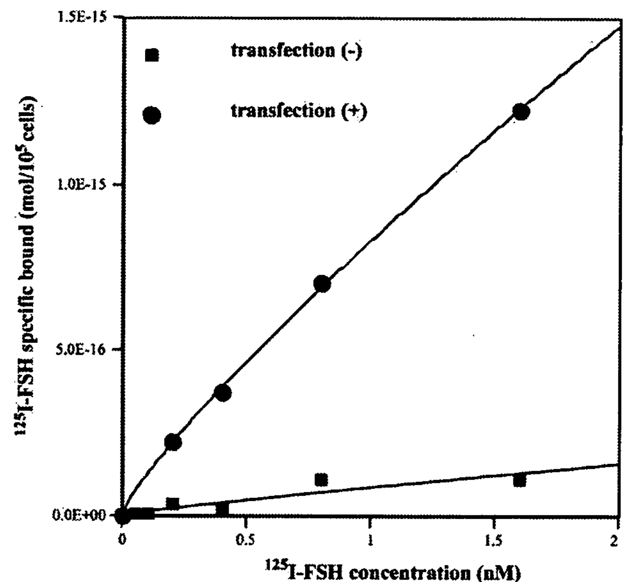

Fig. 3. ^{125}I-FSH specific binding to COLO320 cells. COLO320 cells were seeded into 96-well (10^5 cells per well) tissue culture plates and allowed to attach overnight. The next day, FSHR was introduced into COLO320 cells by the above-mentioned method. Forty-eight hours after exposure to adenoviruses, RRA was performed (●). As a control, the cells were incubated with the virus free culture medium (■).

On day 0, FSH stimulated estradiol production in granulosa cells, but the production of estradiol decreased with time in culture and on day 5, the significant response of estradiol production was not observed by FSH stimulation (Fig. 2).

Expression of Functional FSHR in COLO320 Cells

To confirm the function of the FSHR transduced by adenoviral vector that we constructed, FSHR was introduced into COLO320 cells. Specific^{125}I-FSH binding was observed in the FSHR transduced COLO320 cells. On the other hand, the labeled FSH did not bind to the cells that lacked the FSHR (Fig. 3). As shown in Fig. 4, RNA blot analysis indicated the expression of the 2.5 kb mRNA of FSHR in total RNA prepared from COLO320 transfected the FSHR gene.

Since there is no aromatase for the production of estrogen in COLO320 cells, the quantity of cAMP production was measured in order to evaluate the function of the introduced FSHR (Fig. 5). Nontransduced COLO320 cells did not produce cAMP in response to FSH because they lacked FSHR. In FSHR transduced COLO320 cells, FSH clearly stimulated cAMP production (1220.0 ± 99.0 pmol/mL).

FSH Binding Assay and RNA Blot Analysis for FSHR Infected Rat Granulosa Cells

Rat granulosa cells were seeded into 96-well (10^5 cells per well) E-C-L coated plates and allowed to attach overnight. Transfection and RRA were carried out as described for COLO320 cells above. The number of receptors was calculated from Scat chard analysis (Fig. 6). Although the number of receptors expressed on the surface of the noninfected cells was 2176.0 ± 1288.8/cell, transfected cells expressed an increase in the number of receptors by the third day 7206.3 ± 2407.1/cell. This increase was statistically significant ($p < 0.001$). As shown in Fig. 7, 2.4 kb of endogenous FSHR mRNA was detected in the total RNA prepared from rat granulosa cells immediately after sacrifice. Subsequently, the FSHR mRNA level was markedly reduced after 72 h. A remarkable increase of the 2.5 kb mRNA from introduced FSHR gene was noted. This increase was detected by the third day.

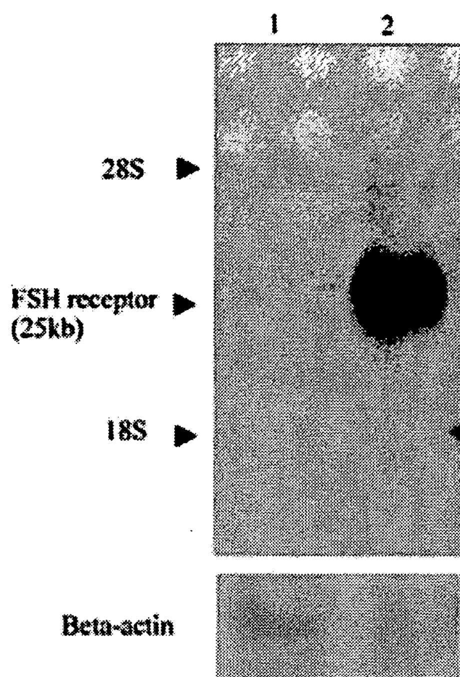

Fig. 4. RNA blot analysis of transduced FSHR gene expression in COLO320 cells. The samples were transferred to a nylon membrane and hybridized to probes for FSHR or beta-actin. The probes of FSHR were obtained by PCR from the murine DNAs. The migrating positions of the 28S and 18S ribosomal RNAs are indicated.

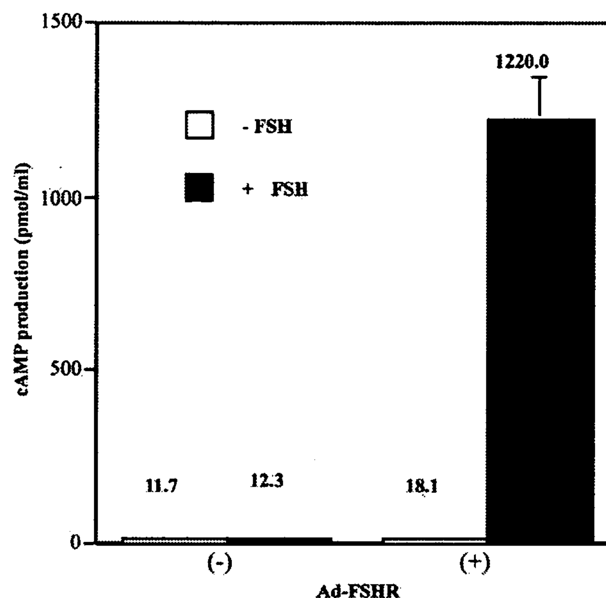

Fig. 5. cAMP production of COLO320 cells transfected the FSHR gene or cells without transfection. Cells were seeded to 96-well plates and incubated for 24 h at 37°C in the presence or absence of 100 mIU/mL FSH. All experiments were performed in the presence of IBMX. The data are mean ± SD of triplicate determinations. Only the cells by which FSHR gene was introduced were highly responsive to FSH.

FSH-Stimulated Estradiol Production from FSHR Transfected Rat Granulosa Cells

Since there is aromatase for the production of the estrogen in granulosa cells, the function of the introduced FSHR gene in the presence of androstenedione (50 ng/mL) as substrates was assessed by measuring the quantity of estradiol production. The amount of estradiol production was significantly higher in the infected cells compared with uninfected cells on the third day of culture (5939.8 ± 2369.1 vs. 191.6 ± 205.5 pg/ml, $p < 0.01$) (Fig. 8).

DISCUSSION

In mammalian species, immature oocytes are capable of undergoing spontaneous nuclear maturation in vitro, and then normal fertilization and cleavage. Unfortunately, embryos derived from in vitro matured oocytes reveal the reduced implantation potential when compared with in vivo matured oocytes.

The establishment of a culture condition that supports cytoplasmic maturation is required for subsequent fertilization. Many culture conditions for IVM of immature oocytes have been developed and published (12).

Janssenswillen *et al.* showed that co-culture with Vero cells improved the in vitro maturation of human GV-stage oocytes (13). Granulosa cells play a crucial role for complete maturation of the oocyte by secreting a wide variety of growth factors. Therefore, co-culture of granulosa cells could promote the synchronous nuclear and cytoplasmic maturation of oocytes. In the cumulus-intact oocytes matured in vitro, the rate of normal fertilization and cleavage were higher than similar rates in cumulus denuded oocytes (14). Dandekar *et al.* reported that 44% of immature oocytes were fertilized after coculture with granulosa cells compared with 20% fertilization rate in immature oocytes cultured without granulosa cells (15). Shramm and Bavister demonstrated that the development into morulae and blastocysts was improved by co-culture with granulosa cells compared with no granulosa cells (16). Granulosa cells obtained form antral follicles rapidly lose the expression of FSHR and steroidogenesis function in primary culture conditions. In this study, we

showed that the number of FSHR on granulosa cells decreased with time in culture. In addition, by the fifth day, the estradiol production was not observed in response to FSH. Therefore co-culture with granulosa cells has a limited role in helping to achieve satisfactory rates of oocyte maturation for clinical application.

In this study, we introduced the FSHR gene into primary rat granulosa cells by adenoviral vector to maintain their response to FSH. We developed the replication-deficient adenoviral vector (AxCAFSHR) expressing FSHR under a constitutive promoter. We showed that COLO320 cells transduced FSHR gene by AxCAFSHR express functional FSHR on their cell surface. The adenovirus vector we constructed was able to efficiently direct the expression of exogenous protein in primary cultured granulosa cells confirmed by the expression of b-galactosidase in more than 80% of the cells.

When the granulosa cells were infected with AxCAFSHR on the first day of culture, the decrease of the expression of the FSHR was significantly restored in the infected cells compared with the uninfected cells on the third day of culture ($p < 0.001$) (Fig. 6). Also we confirmed the strong expression of the transduced FSHR gene in the infected cells by Northern blot analysis (Fig. 7). The uninfected cells had almost completely lost the expression of endogenous FSHR gene by the third day of culture. Furthermore, the amount of estradiol production was significantly higher in the infected cells than in the uninfected cells on the third day of culture (Fig. 8).

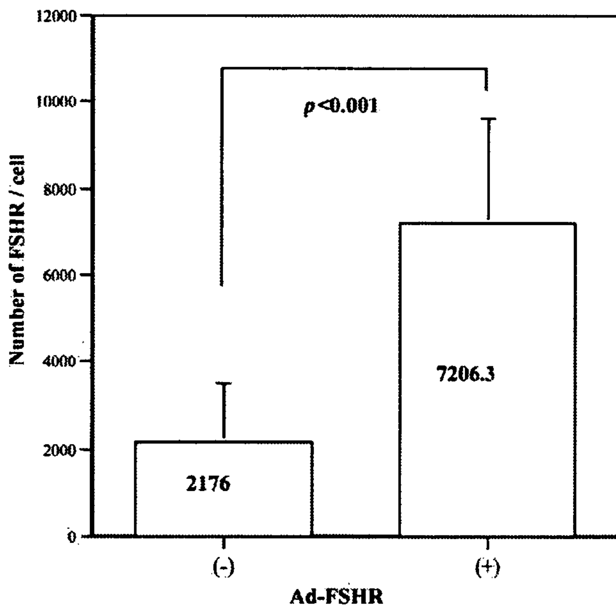

Fig. 6. Number of FSHR on rat granulosa cell on the third day of culture. The FSHR gene was introduced into cultured granulosa cells on the first day of culture, and then FSHR expression was assayed by RRA 48 h later.

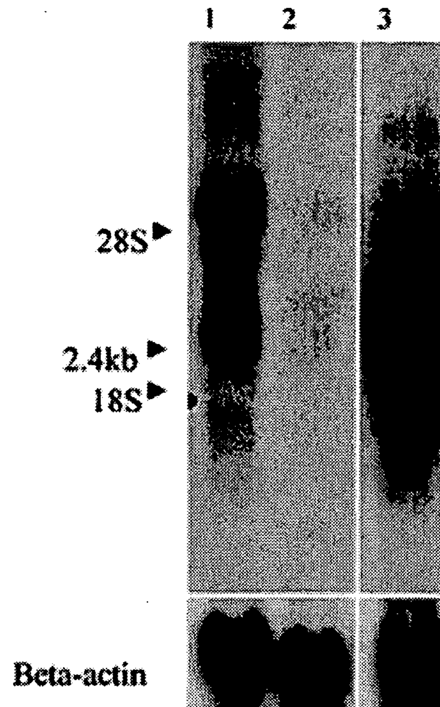

Fig. 7. RNA blot analysis of FSHR gene expression in the cultured rat granulosa cells. Lane 1, rat granulosa cells cultured in vitro for 0 h; lane 2, 72 h; lane 3, rat granulosa cells transduced the FSHR gene. The 2.4 kb band indicates endogenous FSHR mRNA and the 2.4 kb indicates mRNA from transduced FSHR. The migrating positions of the 28S and 18S ribosomal RNAs are indicated.

FSH induces the expression of aromatase and the production of estradiol in granulosa cells. cAMP is the principal mediator of FSHR signals. Indeed, 8-bromo-cAMP (Br-cAMP) is able to induce the expression of aromatase by itself (17). Therefore the function of granulosa cells could be maintained by the addition of Br-cAMP to the culture medium. But cAMP is not solely responsible for FSHR signal transduction (18). Hence we introduced the FSHR gene into granulosa cells to retain their function.

This is the first report to demonstrate that the primarily cultured granulosa cells could be efficiently transduced with FSHR gene by the adenovirus vector. The granulosa cells transfected with FSHR gene maintained the expression of FSHR on their cell surface and the response to FSH stimulation. Our

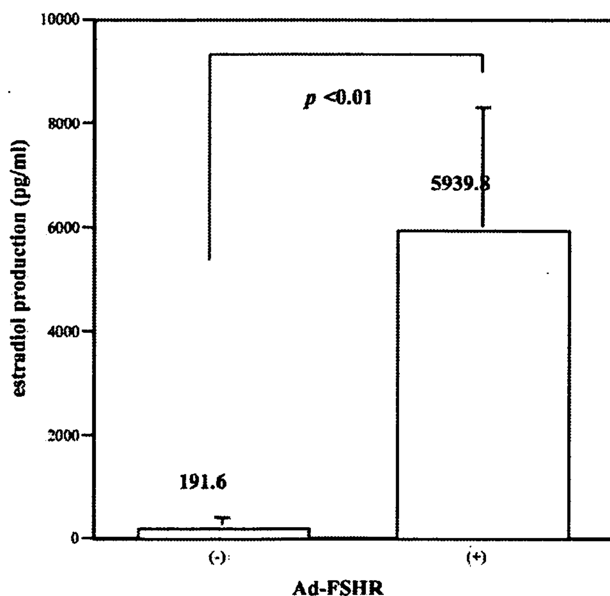

Fig. 8. Estradiol production of rat granulosa cells transfected FSHR gene. Ad-FSHR was introduced into cultured granulosa cells on the first day of culture, FSH and androstenedione were added to the medium 48 h after transfection. The medium was collected at 48 h after the addition of hormones and the estradiol was measured by radioimmunoassay.

study demonstrates that gene therapy can be used to sustain granulose cell function in vitro. This may have reproductive implications. So we suggest that co-culture of the immature oocytes with FSHR transduced granulose cells would improve their developmental competence.

ACKNOWLEDGMENTS

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Association Between Single Nucleotide Polymorphisms of Drug Resistance-associated Genes and Response to Chemotherapy in Advanced Ovarian Cancer

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Abstract. *Background: Single nucleotide polymorphisms (SNPs) may show clinicopathological importance as prognostic markers. This study examined the association of SNPs and the expression of drug resistance-associated markers with response to chemotherapy in advanced ovarian cancer (stages III and IV) patients. Materials and Methods: SNPs were analyzed for MDR1, MRP1, MRP2 and LRP in 60 advanced ovarian cancer patients. The protein expression of each factor was analyzed by immunohistochemistry in all patients. Results: As a result of examining the relevance of SNP genotypes to the response to chemotherapy, a significant relevance (p=0.01) was observed regarding MRP1 exon-17 SNP (G2168A) involving amino acid substitution. No significant relationship was observed between protein expression and the response to chemotherapy or disease-free survival time. Conclusion: Analysis of drug resistance gene polymorphism appears to be an indicator of the response to chemotherapy in advanced ovarian cancer.*

Ovarian cancer has the highest mortality rate of all female genital tumors. Since advanced ovarian cancer, in which the tumor cannot be completely removed surgically, accounts for 75% of cases, the efficacy of postoperative chemotherapy becomes an important factor affecting prognosis. Although the prognosis for ovarian cancer has improved with platinum-based chemotherapy and the appearance of taxane drugs, the 5-year survival rate for advanced ovarian cancer remains low at 20-40% (1). Drug resistance to anticancer drugs greatly affects the prognosis. There are various drug resistance mechanisms, such as a decrease in the intracellular drug level due to the natural pumping mechanism which eliminates drugs from the cells, the inhibition of apoptosis, and the abnormality of genes which normally take part in DNA repair.

The members of the ATP-binding cassette transporter (ABC-transporter) superfamily, typically MDR, MRP and LRP, have the function of pumping intracellular drugs out of the cells, and their activity is dependent on ATP (2-4). These proteins have attracted attention because it has been shown that they are present in the digestive tract, the blood vessel walls and at the blood-brain barrier, absorbing and metabolizing various drugs, and acting as resistance factors to anticancer drugs (3, 5-7).

There are indications that MDR1 may be associated with resistance to paclitaxel (8-12) and that MRP1 and MRP2 may be associated with resistance to platinum-containing drugs (13, 14), although there are reports that the expressions of these resistance factors do not affect prognosis (2, 15, 16). LRP is also linked to multidrug resistance in cancer cell lines and has been reported to be to be an independent prognostic factor of response to chemotherapy and survival (15, 17).

In this study, the genetic polymorphisms for MDR1, MRP1, MRP2 and LRP in advanced ovarian cancer patients and the protein expressions in tumor tissues were analyzed, in order to examine their relevance in terms of the response to chemotherapy and prognosis.

Materials and Methods

Patients and specimens. The clinicopathological backgrounds of the subjects in this study are shown in Table I. The subjects comprised 60 FIGO stage III and IV primary ovarian cancer cases, who had undergone treatment in our hospital from 1990 to 2000. The subject group presented no other form of cancer. Fifty cases were at stage III and ten cases were at stage IV of the disease. There were 49 serous adenocarcinoma cases and eleven endometrioid adenocarcinoma cases. The diagnosis was based on conventional morphological examination of paraffin-embedded specimens by pathologists. Clear cell adenocarcinoma, mucinous adenocarcinoma

Abbreviations: MDR1, multidrug resistance gene-1; MRP, multidrug resistance-associated proteins; LRP, lung resistance protein.

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Key Words: Drug resistance gene, MRP1, SNP, ovarian cancer, chemotherapy.

Table I. *Clinicopathological variables and protein expression of drug resistance genes.*

	No.	MDR1+(%)	MRP1+(%)	MRP2+(%)	LRP+(%)	Significance
All cases	60	18 (30.0)	23 (38.3)	29 (48.3)	14 (23.3)	
Stage						
III	50	13 (26.0)	18 (36.0)	23 (46.0)	10 (20.0)	NS
IV	10	5 (50.0)	5 (50.0)	6 (60.0)	4 (40.0)	
Histiotype						
serous	49	12 (24.5)	17 (34.7)	23 (46.9)	9 (18.4)	NS
endometrioid	11	6 (54.5)	6 (54.5)	6 (54.5)	5 (45.5)	
Residual tumor						
≤2 cm	22	8 (36.4)	9 (40.9)	13 (59.1)	8 (36.4)	NS
>2 cm	38	10 (26.3)	14 (36.8)	16 (42.1)	6 (15.8)	

NS: not significant.

and germ cell tumor cases were excluded, because their cytobiological characteristics, such as reactivity to chemotherapy, appeared different. Primarily all the patients were treated with debulking surgery and five cycles of postoperative chemotherapy, which consisted of a platinum-based regimen in 40 patients and a taxane-platinum combination in 20 patients (Table II). After initial surgery, 22 cases had a remaining tumor diameter of 2 cm or less, while in 38 cases the remaining tumor diameter was over 2 cm. The response to induction chemotherapy was assessed by clinical and/or radiographic evaluation according to the WHO criteria. Of 43 patients assessable for response to chemotherapy, nine (21%) achieved a complete response, 17 (40%) achieved a partial response, six (14%) had no change and eleven (26%) showed progressive disease (Table II).

Immunohistochemistry. The protein expressions were studied with gene-specific antibodies for MDR1 (C-19), MRP1 (C-20), MRP2 (H-17) and LRP (C-1014) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the initial surgical specimens. For each sample, a peroxidase-conjugated secondary antibody identified the binding of the primary antibody. Staining was carried out using a DAKO LSAB+Kit (DAKO, Glostrup, Denmark). Positive and negative controls played an appropriate role in each of the antibodies. Stained tissue specimens were examined under a microscope in three high-power fields, in order to determine the degree of staining of each factor. In concordance with former publications, the samples were classified as positive for the expression if >5% of the tumor cells showed immunoreactivity for each factor (2, 15, 16, 18).

Genotyping. DNA was extracted from the tumor tissue in order to analyze the single nucleotide polymorphisms (SNPs) of each factor. SNPs with a minor allele frequency of 5% or upward were analyzed by reference to the JSNP database (19), which is a Japanese public database (http://snp.ims.u-tokyo.ac.jp/index_ja.html), or to past reports of the polymorphism of each factor (20). All PCRs were performed on a Perkin Elmer GeneAmp 9700 system and the presence of amplicons was checked on agarose gels. A single nucleotide primer extension assay was carried out to analyze SNP using a SNaPshot Kit (Applied Biosystems, Foster City, CA, USA). The extended primers were analyzed on an ABI 3100 (Applied Biosystems). This study was approved by the Niigata University Human Investigation Committee, Japan.

Table II. *Chemotherapy regimens and response to chemotherapy.*

	No. of cases
Chemotherapy regimen	
platinum-based	40
taxane+platinum	20
Response to chemotherapy	
CR	9
PR	17
NC	6
PD	11
DFS (range in months)	25.8 (3-166)

CR: complete response, PR: partial response, NC: no response, PD: progressive disease, DFS: disease-free survival.

Table III. *Relationship between the expressions of MDR1, MRP1, MRP2 and LRP and the prognosis of patients.*

Gene	Protein expression	No.	Response to chemotherapy (CR+PR)/(NC+PD)	Disease-free survival (months) (mean±SD)	Significance
MDR1	-	42	23/13	33.0±31.2	NS
	+	18	4/4	26.4±28.7	
MRP1	-	37	18/9	36.9±28.4	NS
	+	23	8/8	24.7±34.2	
MRP2	-	31	20/10	28.3±31.2	NS
	+	29	6/7	32.0±31.2	
LRP	-	46	22/12	32.8±41.5	NS
	+	14	5/5	19.4±23.2	

NS: not significant (p-value>0.05).
CR: complete response, PR: partial response, NC: no response, PD: progressive disease.

Statistical analysis. StatView version 4.5 (Abacus Concepts Inc., Berkley, CA, USA) stastical software was used. Qualitative variables were analyzed using Fisher's exact two-tailed test or the Chi-squared test, as appropriate. Disease-free survivals were measured in months

Table IV. *Genotypes and allele frequencies of MDR1, MRP1 and MRP2 genes in Japanese subjects.*

Gene location	Nucleic acid change	Amino acid substitution in this study	Allele frequencies (major alleles) in other subject*	Allele frequencies (major alleles)
MDR1				
Promotor 1	T/C	-	0.908	-
Promotor 2	A-41aG	-	0.883	0.927
exon-12	T1236C	Gly412Gly	0.633	0.615
exon-26	G/A	Ile1144Ile	0.500	-
exon-28	A/G	-	0.742	-
MRP1				
exon-8	T825C	Val275Val	0.600	0.625
exon-9	T1062C	Asn354Asn	0.567	0.646
exon-13	T1684C	Leu562Leu	0.750	0.802
exon-16	C2007T	Pro669Pro	0.950	0.917
exon-17	G2168A	Arg723Gln	0.917	0.927
exon-28	G4002A	Ser1334Ser	0.883	0.844
MRP2				
Promotor	C-24T	-	0.867	-
exon-10	G1249A	Val417Ile	0.925	0.875
exon-28	C3972T	Ile1324Ile	0.758	0.781

*Ito S. *et al.* 2001 (ref. 20).

from the date of surgery to a reported relapse. Survival curves were determined using the Kaplan-Meier method, and differences in survival between subgroups were compared using the log-rank test. *P*-values of less than 0.05 were regarded to be significant. All reported *p*-values were two-sided.

Results

The relationship between the protein expression for each drug resistance factor analyzed and the clinicopathological factors are shown in Table I. According to the immunohistochemical staining, the frequencies of positive reactions to protein expression were 30.0% for MDR1, 38.3% for MRP1, 48.3% for MRP2 and 23.3% for LRP. The expression frequencies for the factors of advanced stage disease were 28.0%, 36.0%, 46.0% and 20.0%, respectively, for the stage III cases, and 50.0%, 50.0%, 60.0% and 40.0%, respectively, for the stage IV cases. That is, it was observed that the expression frequencies of MDR1, MRP1, MRP2 and LRP tended to be high at stage IV, but no significant difference was observed. No relevance was observed in terms of the degree of surgical achievement.

The relationship between the expression of each drug resistance factor and the therapeutic response to chemotherapy was examined. The response to induction chemotherapy was assessed by clinical and/or radiographic evaluation. The cases with evaluable lesions (by computed tomography) after initial surgery were classified into two groups; the group in which chemotherapy was effective (CR/PR) and the group in which it was not (NC/PD), in order to examine whether there was any difference in the expression of each factor. No correlation was observed between the protein expression and response to chemotherapy in any drug resistance factors, although the response to chemotherapy tended to be low in the positive group (Table III). For the cases without evaluable lesions after initial surgery, the correlation between the disease-free survival time and the protein expression for each factor was examined. No significant relationship was observed between the protein expression and the disease-free survival time (Table III).

Sixteen SNPs for MDR1, MRP1, MRP2 and LRP were analyzed in order to determine their genotypes. The SNP analyses were conducted upon selecting polymorphisms in the promoter area and exon polymorphisms, which may affect protein expression. Two SNPs in the LRP gene were excluded from this study, because there were no variants in the subjects analyzed. Table IV shows the 14 SNPs analyzed in this study. The minor allele frequencies for each polymorphism were between 7.5% and 50%, and no significant differences were observed in comparison with the allele frequencies previously reported (20).

The relationship between the protein expression of each drug resistance factor and the relevant SNP genotype was examined. For each SNP genotype, wild-type homo was taken as the *w* group and minor allele homo and hetero as the *m* group, and the difference in the frequency of positive reactions to the protein expression of each factor was examined and compared between the two groups. No difference in protein expression by SNP genotype was observed for any factor (data not shown). The relevance of the SNP genotype for each drug resistance factor to the response to chemotherapy and disease-free survival time was

Table V. *Relationships between genotypes of drug resistance genes and prognosis of patients.*

Gene	Location	Genotype	No.	Response to chemotherapy	Significance	Disease-free survival (months) (mean±SD)	Significance
MDR1	Promotor 1	*w*	50	21/13	NS	35.0±50.8	NS
		m	10	5/4		23.2±30.7	
	Promotor 2	*w*	48	20/13	NS	24.7±31.2	NS
		m	12	6/4		33.4±46.8	
	Exon-12	*w*	26	14/7	NS	21.1±20.4	NS
		m	34	12/10		30.5±32.7	
	Exon-26	*w*	14	9/6	NS	34.4±34.2	NS
		m	46	17/21		23.9±25.3	
	Exon-28	*w*	34	16/7	NS	21.7±26.1	NS
		m	26	10/10		34.4±38.5	
MRP1	Exon-8	*w*	20	12/6	NS	29.2±35.5	NS
		m	40	14/11		26.0±36.6	
	Exon-9	*w*	22	10/6	NS	32.6±36.5	NS
		m	38	16/11		27.8±44.0	
	Exon-13	*w*	33	14/8	NS	23.5±37.0	NS
		m	27	12/9		31.0±34.3	
	Exon-16	*w*	54	22/15	NS	34.5±31.5	NS
		m	6	4/2		46.4±41.1	
	Exon-17	*w*	59	18/17	p=0.011	26.3±35.2	NS
		m	11	11/0		24.4±13.9	
	Exon-28	*w*	48	21/13	NS	33.2±40.5	NS
		m	12	5/4		22.0±20.5	
MRP2	Promotor	*w*	46	18/14	NS	29.0±38.8	NS
		m	14	11/5		23.2±20.7	
	Exon-10	*w*	52	12/15	NS	26.7±31.2	NS
		m	8	7/4		24.4±46.8	
	Exon-28	*w*	35	24/17	NS	25.1±32.4	NS
		m	25	18/11		29.5±31.7	

w: wild-type/wild-type.
m: wild-type/mutant and mutant/mutant.
NS: not significant (p-value >0.05).
CR: complete response, PR: partial response, NC: no response, PD: progressive disease.

examined (Table V). As a result of examining the relevance of the SNP genotypes to the effects of chemotherapy in 44 cases with evaluable lesions after initial surgery, a significant relevance was observed regarding MRP1 exon-17 SNP (G2168A) involving amino acid substitution. In the group (group *m*) with the minor allele for MRP1 exon-17 SNP (G2168A), the response to chemotherapy was 100%, whereas in the *w* group, the response rate was 51.4%, which was significantly low (p=0.011). No significance was observed in terms of the relevance of the SNP genotype to the disease-free survival time in the cases without evaluable lesions as a result of initial surgery.

Discussion

It was demonstrated that a genotype of SNP in exon-17 of the MRP1 gene, but not the protein expression of MRP1, was relevant to the response to chemotherapy. To the best of our knowledge, this study is the first to demonstrate the linkage between MRP1 gene polymorphism and therapeutic efficacy for ovarian cancer patients.

The members of the ABC-transporter superfamily, MDR1, MRP1, MRP2 and LRP, are known to play important roles *in vitro* in eliminating drugs, including anticancer drugs and hormones from the cells (3, 14, 21). It is presumed that the acquisition of drug resistance derives from the elimination of anticancer drugs from cells, a mechanism facilitated by the enhanced expression of these factors. Indeed, it has been reported that the correlation of these factors with the efficacy of chemotherapy and prognosis was observed in lung cancers (22), sarcomas (18) and blood disorders such as acute mycloid leukemia (6, 23). In ovarian cancer cases, the reports are contradictory; some reports assert the correlation of these factors with prognosis, while others assert the opposite (2, 15, 24), indicating a dichotomy of opinions regarding the clinical meaning of the factors.

As a result of the rapid progress of gene analysis techniques in recent years, the sequences of many genetic polymorphisms

have been added to public databases and have become available (19, 20, 25). Knowledge of the correlation between polymorphisms and diseases is expected to enable the identification of causative genes and, thus, their to individualized therapies.

In this study, through the analysis of genetic polymorphisms (SNPs) of the members of the ABC-transporter superfamily in Japanese advanced ovarian cancer patients and the analysis of the expression of proteins, which are the gene products of genetic polymorphisms, the relationships between SNPs and protein expression, the associations of genetic polymorphisms and protein expression with response to chemotherapy and prognosis were examined.

The examination of the relevance of the protein expression of each factor, as assessed by immunohistochemical staining, to the response to chemotherapy and progression-free survival time showed no significant correlation for any factor analyzed in this study. It has been reported that MDR1 shows resistance to taxol *in vitro* by eliminating taxane drugs from cells, even when it manifests itself in extremely small quantities (10). Also, it has been reported that the expression of MDR1 mRNA was observed in 36% cases of ovarian cancer patients; in those cases, the progression-free survival times were short (26). MRP1 has been reported to be overexpressed in ovarian cancer patients and the high relative mRNA levels of MRP1 expression were significantly correlated with a short period of progression-free survival (27). Although there are few reports as to the expression frequency of MRP2 in ovarian cancer cases, it has been reported that the expression of MRP2 *in vitro* was very frequently observed in cells resistant to cisplatin (26). As for LRP, it has been reported that it is relevant to the efficacy of chemotherapy in breast cancer cases (28) and in blood diseases, such as acute myelogenous leukemia (AML) and multiple myeloma (6, 29). Also, it has been reported that LRP serves as a prognosis indicator in ovarian cancer cases (15).

However, there are reports where no relevance of any of the drug resistance factors with the efficacy of chemotherapy or the prognosis of ovarian cancer cases could be detected (2). Thus, opinions differ as to the clinical significance of these factors. Here, no significant relevance between the protein expression of each factor to the outcome of chemotherapy and prognosis was revealed. Thus, an accurate prediction of the therapeutic effects of chemotherapy, based solely on the evaluation of protein expression by immunohistochemical staining, seems difficult to achieve. If a more sensitive method, such as RT-PCR, were used, a significant correlation might be observed.

Regarding the SNP of each factor, it has been reported that the MDR1 exon-26 polymorphism (C3435T) is related to the expression of MDR1 in the digestive tract (30, 31). The polymorphism is reported to be associated with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (5). Also, there is a report regarding a significant correlation between clinical complete response to preoperative chemotherapy for breast cancer patients and the SNP genotype (32). On the other hand, other reports state that no relationships were observed between the expression and prognosis in digestive organ cancer cases (31) and blood diseases, such as acute lymphocytic leukemia (7). Although the SNPs of other ABC-transporter family member genes and their phenotypical consequences and pharmacological implications have been reported (21, 33), no report exists regarding the relationship between those SNPs and the therapeutic efficacy and prognosis of ovarian cancer patients.

In this study, the relevance of the SNPs of MDR1, MRP1 and MRP2 to protein expression, the response to chemotherapy and disease-free survival time were examined. As a result, no relationship between any SNP genotype and protein expression was observed. Thus, it is presumed that the promoter and exon SNP analyzed in this study had no effect on protein expression. In examination of the relevance of SNPs to the response to chemotherapy and prognosis, only a SNP in exon-17 of MRP1 was observed to be relevant to the response to chemotherapy. The MRP1 exon-17 SNP is a polymorphism involving amino acid substitution, but no relevance to protein expression was determined. Therefore, it is possible that the polymorphism may affect the function of the gene products instead. Exon-17 in the MRP1 gene encodes two nucleotide-binding domains (NBDs) existing in MRP1. It is presumed that ATP binds with these NBDs, thus playing an important role in the pumping action of proteins belonging to the ABC-transporter superfamily. It has been shown, experimentally, that a mutation induced artificially in these NBDs inhibits their function (4). On the basis of these reports, the significant correlation with the response to chemotherapy found in this study seems to suggest the possibility that exon-17 G2168A (Arg723Gln) polymorphism is relevant to the change in the function of MRP1, although the precise mechanism requires further examination.

In conclusion, in this study no relevance of any drug resistance factor (MDR1, MRP1, MRP2 and LRP) to protein expression, the response to chemotherapy, or the disease-free survival time was observed. Regarding SNP analysis, it was observed that the SNP in exon-17 of MRP1 was relevant to the response to chemotherapy. Whenever the relevance of drug resistance factors to the reactivity to drugs and prognosis is examined, the changes in function, including gene mutations, as well as the expression levels, should be considered. If it becomes possible to find SNPs which can point to the outcome of anticancer drug therapy, individualized therapy, including the possibility to choose the drugs, will become possible. For the elucidation of the drug resistance mechanism, further investigations into the relationhip between SNP and protein function would appear important.

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Case Report

Metastatic choriocarcinoma successfully treated with paclitaxel and carboplatin after interstitial lung disease induced by EMA-CO

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Abstract

Background. Well-established first-line chemotherapy (such as EMA-CO) is extraordinary active for gestational choriocarcinoma. However, it causes very serious situation once drug-induced acute interstitial lung disease occurs during the treatment.

Case. A 31-year-old Japanese woman with metastatic choriocarcinoma was treated with EMA-CO as an initial chemotherapy regimen for seven cycles. Her beta-hCG dropped from 13,087 ng/ml to 2.2 ng/ml. At 11 days after 7th cycle of EMA-CO treatment, however, she developed respiratory failure, and was diagnosed as having EMA-CO-induced interstitial lung disease with bilateral ground-glass opacity on CT scan, and the examination of the bronchoalveolar lavage fluid. After high-dose steroid therapy, symptoms and ground-glass opacity on CT scan were remarkably improved. She then commenced a regimen of carboplatin (AUC 5) and paclitaxel (180 mg/m^2). After completing 8 cycles, her beta-hCG dropped to <0.2 ng/ml. Three additional cycles were administered and the patient remained clinically free of disease, with normal beta-hCG levels for 11 months.

Conclusions. Paclitaxel and carboplatin combination is active and appears to be a viable alternative to EMA-CO combination chemotherapy in metastatic choriocarcinoma even after EMA-CO-induced interstitial lung disease.
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Keywords: Metastatic choriocarcinoma; Drug-induced interstitial pneumonitis; EMA-CO; Paclitaxel; Carboplatin

Introduction

Gestational choriocarcinoma is one of the most highly curable forms of adult malignancy, largely due to the exceptional sensitivity of this tumor to chemotherapy. Well-established first-line chemotherapy (such as EMA-CO [1]) is highly active for gestational choriocarcinoma. However, it causes very serious situation once drug-induced acute interstitial lung disease occurs during EMA-CO treatment for gestational choriocarcinoma. A cisplatin-based regimen such as EMA-EP is generally agreed to be the most appropriate second line regimen [2]. BEP (bleomycin, etoposide, cisplatin) [3], VIP (etoposide, ifosfamide, cisplatin), and ICE (ifosfamide, carboplatin, etoposide) [4] are accepted as the third line and all produce cures in these settings. The use of these second and third line regimes is hesitated after EMA-CO-induced interstitial lung disease.

Here we report a case with metastatic choriocarcinoma treated successfully with paclitaxel and carboplatin after EMA-CO-induced interstitial lung disease.

Case report

A 31-year-old Japanese woman, gravida 1, para 1-0-0-1, whose last pregnancy resulted in a normal vaginal delivery in May 2000, presented to her gynecologist in December, 2003 with amenorrhea since September 6, 2003. Transvaginal ultrasound examination showed no abnormality in the endometrium and bilateral adnexae. Her serum beta-hCG level was found to rise to 13,087 ng/ml, and she was referred to our hospital. Computed tomography (CT) scan revealed multiple tumor formation in the lung 5 × 4 cm in size, and liver tumors maximal 6 cm in diameter (Figs. 1A, B). Pelvic and brain

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