Experimental characterization of recurrent ovarian immature teratoma cells after optimal surgery

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Abstract. Minimal optimal surgery without chemotherapy is often performed for patients with ovarian immature teratoma, which frequently occurs in young women who hope for future pregnancies. If tumors recur after the operation, anticancer drug chemotherapy is often administered, although few studies have highlighted differences between the recurrent and the primary tumor cells. Therefore, we have established experimental animal models of recurrent ovarian immature teratoma cells after optimal surgery and characterized the anticancer drug sensitivity and antigenicity of the recurrent tumors. Surgically-excised tumor cells of a grade II ovarian immature teratoma were cultured *in vitro* and transplanted into nude mice to establish stable cell lines. Differential drug sensitivity and antigenicity of the tumor cells were compared between the primary and the nude mouse tumors. Nude mouse tumor cells showed a normal 46XX karyotype. Cultured primary cells showed a remarkably high sensitivity to paclitaxel, docetaxel, adriamycin and pirarubicin, compared to peritoneal cancer cells obtained from a patient with ovarian adenocarcinomatous peritonitis. The drug sensitivity of teratoma cells to 5-fluorouracil, bleomycin or peplomycin was also significantly higher. However, there was no significant difference in sensitivity to platinum drugs between the primary teratoma and the peritoneal adenocarcinoma cells. As for nude mouse tumor cells, sensitivity to 12 anticancer drugs was significantly lower than that of the primary tumor cells, while there was little difference in sensitivity to carboplatin or peplomycin between the primary and nude mouse tumor cells. Flow cytometry showed that the expression of smooth muscle actin (SMA) significantly decreased in nude mouse tumor cells when compared to cultured primary cells. In conclusion, ovarian immature teratomas with normal karyotypes have a malignant potential to recur after minimal surgery. During nude mouse transplantation, SMA-overexpressing cells appeared to be selectively excluded and nude mouse tumor cells were less sensitive to the majority of anticancer drugs than the primary tumor cells. These results indicate that after optimal surgery for ovarian immature teratoma, recurrent cells can be more resistant to anticancer drugs than the primary tumors. Therefore, it is likely that adjuvant chemotherapy lowers the risk of ovarian immature teratomas recurring after optimal surgery. BEP and PBV regimens are frequently given to teratoma patients. However, paclitaxel/carboplatin or docetaxel/carboplatin, which are the most effective chemotherapy treatments for epithelial ovarian cancer patients, are considered to be an alternative regimen, especially in the prevention of reproductive toxicity.

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Introduction

Ovarian immature teratoma is one of the malignant ovarian germ-cell tumors and prognosis is dependent on clinical staging and histopathological grading (1-3), although in general, the anticancer drug sensitivity of ovarian immature teratoma cells is usually better than that of epithelial ovarian cancer cells. The following chemotherapeutic regimens have been reported to show significant clinical responses: vincristine, dactinomycin and cyclophosphamide (VAC) (4-8); vincristine, actinomycin-D and cyclophosphamide (VAC) (9,10); cisplatin, vinblastine and bleomycin (PVB) (11,12) as well as bleomycin, etoposide and cisplatin (BEP) (13,14). Since ovarian immature teratoma frequently occurs in young women, unilateral salpingo-oophorectomy as an optimal minimal surgery is usually performed, with the exception of patients who have advanced tumors or those who do not hope for a future pregnancy (1-3). However, anticancer drug chemotherapy is required if macroscopic tumor tissue remains at the site of surgery or if the tumor recurs.

If a patient hopes to become pregnant in the future, chemotherapy drugs with a high ovarian toxicity cannot be used. VAC, PVB or BEP have become the most frequently given drug regimens for malignant ovarian germ-cell tumors. However, cyclophosphamide (15-17) and etoposide (18-20) are reported to have strong ovarian toxicity. Furthermore,

there are no reports thus far that examine whether VAC, PVB or BEP are the best regimens for ovarian immature teratomas.

To establish the optimal anticancer drug chemotherapy for malignant ovarian immature teratomas and, to examine whether PVB or BEP are effective and safe regimens, it was necessary to establish stable cell lines derived from ovarian immature teratoma cells for *in vitro* and *in vivo* research. Ovarian immature teratomas have characteristic immature and/or mature ectodermal, endodermal and mesodermal tissues. It is highly possible, therefore, that immature teratoma cells that recur after optimal surgery are rich in selected tumor cells with a higher proliferative activity *in vivo*. However, the characterization of such cells has yet to be described. Recurrent immature teratoma cells after optimal surgery are thought to have the ability to form tumors in nude mice when the cultured tumor cells are transplanted. Although numerous cell lines have previously been established from malignant ovarian tumors, there are few reports of cell lines cultured from ovarian immature teratomas. Moreover, cultures of immature teratoma cell lines suitable for transplantation into nude mice are very rare (21). In this study, we established a cell line from ovarian immature teratoma cells that was capable of forming tumors in nude mice and investigated the differential anticancer drug sensitivity and antigenicity of the primary and nude mouse tumor cells.

Materials and methods

Patient and surgical treatment. An 18-year-old nulligravidous high school student came to our hospital complaining of severe lower abdominal pain. Preoperative ultrasound and magnetic resonance (MR) imaging examinations showed a pedicle torsion of a newborn-head-sized solid ovarian tumor and the patient had elevated levels of serum tumor markers, findings suggestive of ovarian immature teratoma (Fig. 1a and b, Table I). Surgery was performed that day. The ovarian tumor was encapsulated in a thick smooth wall, without adhesion, and unilateral salpingo-oophorectomy was performed without rupture of the tumor. No additional surgical resection was required because the patient was young. Macroscopic findings showed a very heterogeneous solid tumor containing various types of tissues from very soft fragile tissues to osteoid hard tissues (Fig. 1c and d).

Peritoneal cytology was negative and the final diagnosis was a clinical stage Ia ovarian tumor even though the precise staging laparotomy for ovarian cancer had not been finished. The postoperative histopathological diagnosis of the tumor was a grade 2 immature teratoma of the right ovary (Fig. 2). The histological findings were in agreement with the macroscopic heterogeneity and showed that the tumor consisted of skin-like, osteoid, intestine-like, and bronchus-like tissues, nerve-like tissues with glial cells and a cerebral ventricle choroid plexus, retina-like tissues, pigmented epithelium, immature to mature cartilage tissues and mature bone tissues (Fig. 2). The elevated serum tumor markers were within the normal range one month post-operatively and, >57 months later, there was no sign of recurrence (Table I).

In vitro culture of immature teratoma cells. After obtaining informed consent from the patient, surgically-resected ovarian tumor cells were cultured *in vitro* as follows: the tissue sample was cut into small fragments with scissors and incubated in medium containing 0.1% w/v collagenase (Wako-Chemicals, Tokyo, Japan), 500 U/ml penicillin (PC) (Gibco BRL, Gaithersburg, MD, USA), 500 μg/ml streptomycin (SM) (Gibco BRL) and 1250 μg/ml amphotericin B (Gibco BRL) for 30 min at 37˚C. The digested tissues were then centrifuged at 1500 rpm for 6 min at 4˚C. Cell pellets were resuspended and cultured in Opti-MEM (Gibco BRL), 5% fetal calf serum (FCS) (Equitech-Bio Inc., Ingram, TX, USA), 100 U/ml PC, 100 μg/ml SM, 250 μg/ml amphotericin B and $3x10^{-7}$ M estradiol. Nude mouse tumor cells were also cultured according to the same procedures as the primary tumor cells.

Histopathology. Tissue specimens from the excised ovarian and the nude mouse tumor were fixed in 10% formalin, dehydrated and embedded in paraffin. Paraffin sections were deparaffinized and then stained with hematoxylin and eosin (Sigma Chemical Co, St. Louis, MO, USA).

Effects of cytokines and growth factors on the morphology of primary tumor cells. Primary tumor cells were seeded in 24-well plates (1 ml/well of Opti-MEM/5% FCS/estradiol, 10,000 cells/ml) and stimulated with one of 25 recombinant human cytokines or growth factors for three weeks. Cell morphology was monitored under a phase-contrast light microscope. Cytokines or growth factors were added to the cultures every three days. The final concentration of recombinant human stimulants were as follows: IL-1-ß, 50 ng/ml (Pepro Tech EC Ltd., London, UK); IL-1ra (interleukin-1 receptor antagonist), 100 ng/ml (Pepro Tech EC Ltd.); IL-4, 100 ng/ml (Genzyme, Cambridge, MA, USA); IL-11, 50 ng/ml (Pepro Tech EC Ltd.); IL-12, 25 ng/ml (Pepro Tech EC Ltd.); IL-13, 100 ng/ml (Pepro Tech EC Ltd.); IL-18, 125 ng/ml (Pepro Tech EC Ltd.); IGF-1, 50 ng/ml (Gibco BRL); IGF-2, 50 ng/ml (Gibco BRL); EGF, 500 ng/ml (Upstate Biotech Inc., NY, USA); bFGF, 50 ng/ml (Progen, Heidelberg, Germany); M-CSF, 2000 U/ml (a kind gift from Welfide Co. Ltd., Osaka, Japan); GM-CSF, 150 ng/ml (a kind gift from Kirin Brewery Company Ltd., Tokyo, Japan); VEGF, 100 ng/ml (Pepro Tech EC Ltd.); IFN-α, 5000 U/ml (a kind gift from Takeda Pharmaceutical Industrial Co., Ltd., Osaka, Japan); IFN-γ, 500 U/ml (a kind gift from Shionogi & Co., Ltd., Osaka, Japan); TNF-α, 250 ng/ml (Genzyme); TGF-ß1, 10 ng/ml (R&D systems, Minneapolis, MN, USA); activin A, 50 ng/ml (R&D systems); RANTES, 100 ng/ml (Pepro Tech EC Ltd.); oncostatin M, 50 ng/ml (Pepro Tech EC Ltd.); leukemia inhibitory factor, 50 ng/ml (Pepro Tech EC Ltd.); prolactin, 250 ng/ml (Pepro Tech EC Ltd.); leptin, 1000 ng/ml (Pepro Tech EC Ltd.) and CD40 ligand, 250 ng/ml (Pepro Tech EC Ltd.).

Limiting dilution analysis. To identify possible stem cell-like tumor cells among the primary ovarian immature teratoma cells, limiting dilution cultures were performed. Two independent cultures of primary tumor cells with >99% viability were seeded at a final cell density of 30, 6 and 1.2 cells/well in 96-well plates. Cultured nude mouse tumor cells were diluted and cultured similarly. For details of limiting dilution culture methods and the calculation of cloning efficiency see

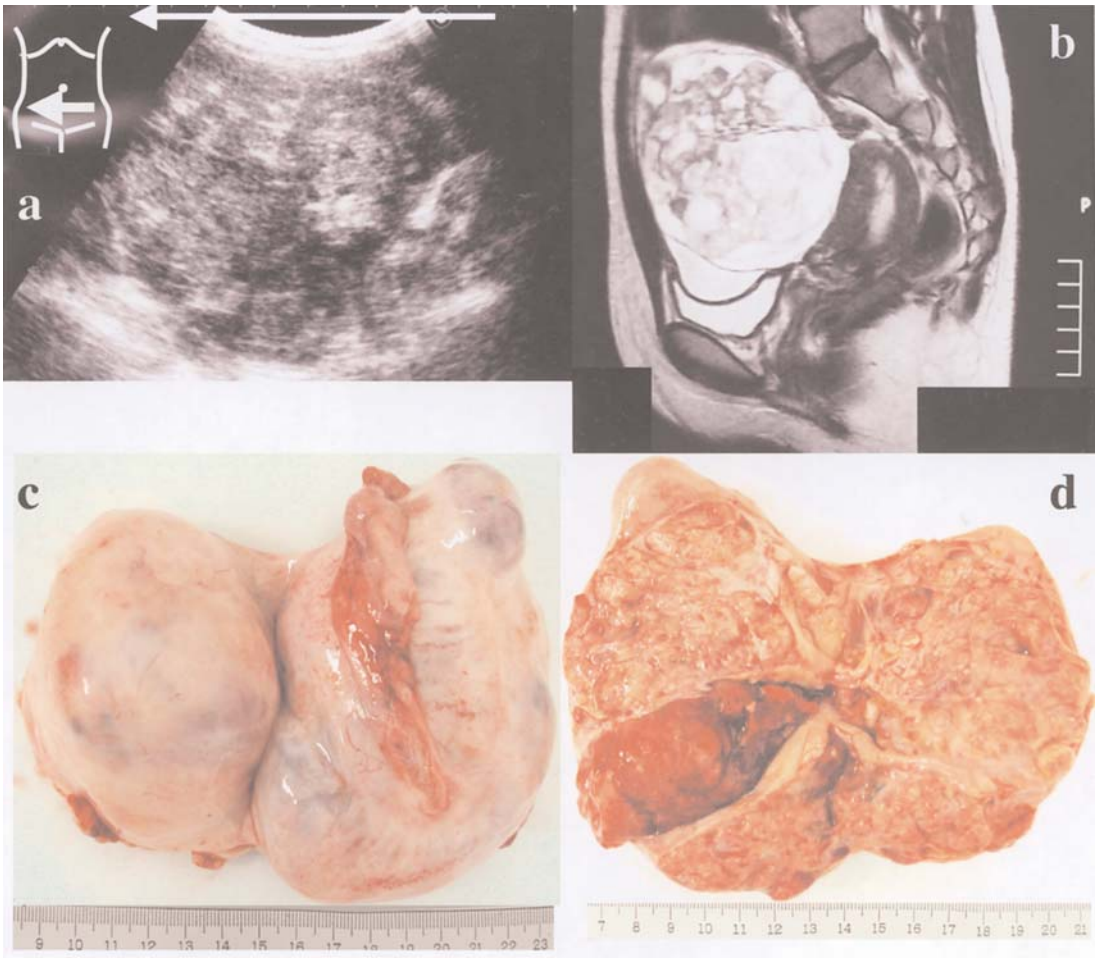

Figure 1. Primary ovarian tumor. (a) Ultrasound and (b) MR images thereof. Preoperative ultrasound examination and MR imaging showed a huge heterogeneous solid tumor. (c) Macroscopic exterior of the resected tumor. The tumor surface was smooth without any adhesion. The tumor was composed of osteoid hard and very fragile tissue. (d) Macroscopic cut surface view of the tumor.

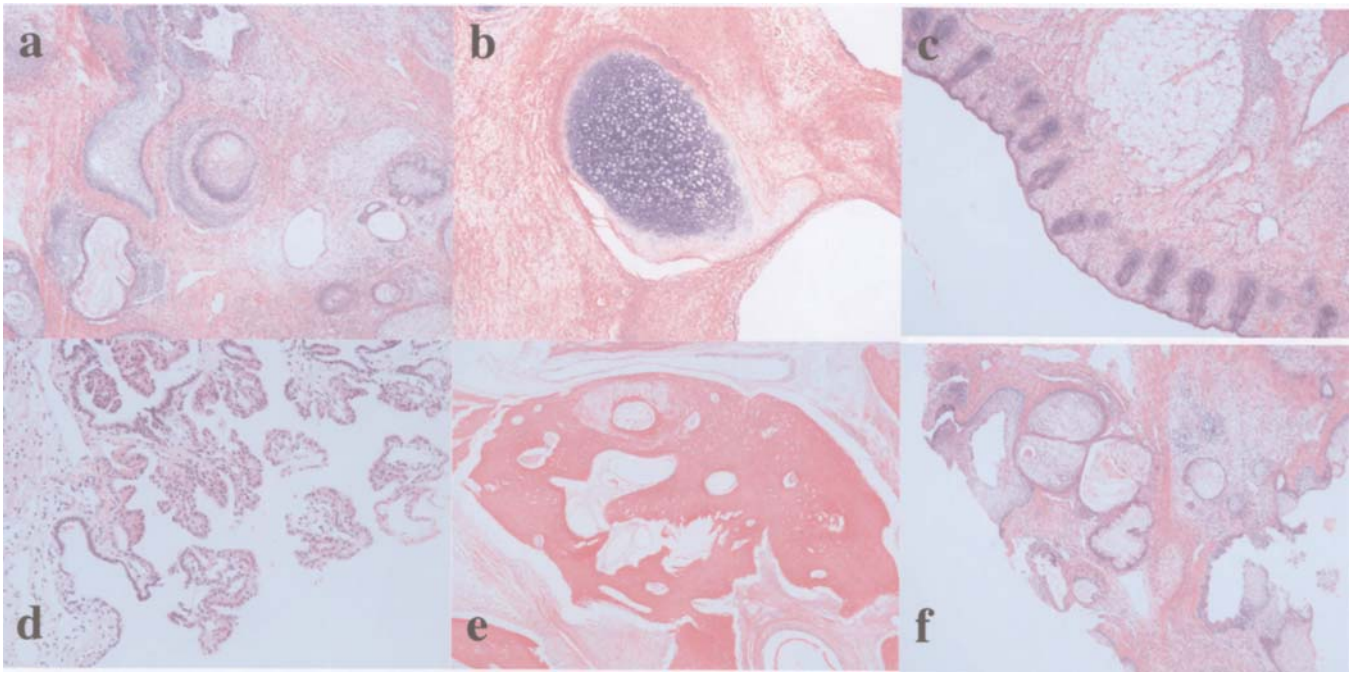

Figure 2. Histopathological findings of the primary tumor tissue. (a-f) In agreement with the macroscopic heterogeneity, the tumor consisted of skin-like, osteoid, intestine-like, bronchus-like, nerve-like and retina-like tissues, pigmented epithelium, cartilage and bone tissues.

Table I. Serum tumor marker levels before and after unilateral salpingo-oophorectomy.

Operative day (0d=surgery)	Normal value	-Id preoperative	+5d postoperative	+48d postoperative
AFP	0-20 ng/ml	222.9	85.1	4.0
CA19-9	0-37 U/ml	201.8	73.7	35.4
SCC	0-1.5 ng/ml	10.1	1.0	0.5
ß-hCG	0-0.5 mU/ml	0.6	<0.5	<0.5
CA125	0-34 U/ml	31.0	n.t.	12.0
CEA	0-5 μg/ml	1.5	n.t.	n.t.

Endocrinological findings (-Id, preoperative) were within normal limits. Estradiol = 37.0 pg/ml, FSH = 2.12 IU/l, LH <0.5 IU/l, TSH = 1.488 ng/ml, free T3 = 2.88 pg/ml, free T4 = 1.06 ng/dl and PRL = 7.67 ng/ml. The patient was positive for the four tumor markers of AFP, CA125, SCC and ß-hCG before the operation but values were within the normal range 48 days after the salpingo-oophorectomy.

our previous study for identifying and establishing stem cell-like cells from human endometrium and endometriotic tissues (22).

Karyotyping analysis. Cytogenetic analysis was performed according to methods reported elsewhere (23,24) with the following modifications: briefly, tumor cell cultures were washed and incubated with 0.1% v/v colcemide (Sigma) overnight. Cells were exposed to a hypotonic solution composed of 3 g/l KCl, 0.2 g/l EGTA and 4.8 g/l Hepes. Cells were spun down and fixed in a methanol solution. G-banding was performed by the Tunis method (25).

Anticancer drugs. All anticancer drugs were kind gifts from pharmaceutical companies. SN38, a major active metabolite of irinotecan-HCl (CPT-11), was obtained from Yakult Co. & Ltd. (Tokyo, Japan). Adriamycin (ADM), 5-fluorouracil (5FU) and mitomycin C (MMC) were provided by Kyowa-Hakko Co. (Tokyo, Japan). Paclitaxel (PTX), carboplatin (CBDCA) and etoposide (VP16) were provided by Bristol-Myers Squip Japan Co. Ltd. (Tokyo, Japan). Pirarubicin-HCl (THP) was provided by Meiji-Seika Co. (Osaka, Japan). Cisplatin (CDDP), bleomycin (BLM) and peplomycin (PEM) were provided by Nihon-Kayaku Co. (Tokyo, Japan). Docetaxel (DOC) was provided by Sanofi-Aventis (Tokyo, Japan). Nedaplatin (NPL) was obtained from Shionogi Co. & Ltd. (Osaka, Japan).

Anticancer drug-sensitivity assays. After obtaining informed consent from the patients, the effects of anticancer drugs on cell growth were assayed. Cells in the log phase were detached with 0.25% trypsin/1 mM EDTA (Gibco-BRL) and cultured overnight in 96-well plates ($5x10^3$ cells/well). On day two, various concentrations of anticancer drugs were added to the cells. On day four, the number of viable cells was evaluated using a cell proliferation assay kit (Dojin, Tokyo, Japan) and expressed as the percentage of viable cells (%) relative to the mean number of viable untreated cells. All experiments were performed in triplicate. Data are expressed as the mean ± SD, and comparative data (n=6) were analyzed by ANOVA.

Nude mouse transplantation. The first nude mouse transplantation was conducted with cultured immature teratoma cells. After five days *in vitro*, the adherent tumor cells were detached in 0.25% trypsin/1 mM EDTA (Gibco BRL) and resuspended in 0.5 ml serum-free OPTI-MEM. Non-adherent tumor cells were collected from the culture medium by centrifugation. The adherent and non-adherent tumor cells were resuspended in media containing 0.5 ml matrigel (Sigma Chemical Co) and transplanted by subcutaneous injection, using a 23G needle, onto the back of three 13-week-old female Balb/c *nu/nu* mice (Nihon Clea, Osaka, Japan). Three months after transplantation nodular tumors grew on the backs of the three mice.

The second nude mouse transplantation was conducted with tumor cells from one of the first mice as follows: tumors were resected and cut into small fragments by scissors and cultured for one week in OPTI-MEM, 5% FCS, 100 U/ml PC, 100 μg/ml SM, 250 μg/ml amphotericin B and $3x10^{-7}$ M estradiol. Cultured cells were collected as described above and resuspended in 1 ml of serum-free OPTI-MEM containing 0.5 ml Matrigel. Cells were injected subcutaneously using a 23G needle onto the back of three 25-week-old female Balb/c *nu/nu* mice. Four to five months after the second transplantation, tumors had grown on the backs of all three mice.

Flow cytometric analyses of cell surface antigens. Cells were detached from the culture flask with 3 mM EDTA in phosphate-buffered saline (PBS), and immunostained according to the following procedure: cells ($3x10^5$) were incubated with an excess of primary antibody for 20 min at 4˚C and washed twice in wash buffer (PBS containing 2% FCS and 0.1% NaN_3). The cells were then incubated in FITC-conjugated goat anti-mouse IgG (H+L) (Dako Japan, Kyoto, Japan) for 20 min at 4˚C and washed twice in wash buffer. Finally, the cells were suspended in 200 μl of wash buffer and analyzed with a FACSCalibur™ (Beckman Coulter Japan, Tokyo, Japan). The primary antibodies were: mouse anti-human CD29 monoclonal antibody (clone TDM29, Cymbus Biotech Ltd., Hampshire, UK); mouse anti-human CD30 monoclonal antibody (clone HRS-4, Immunotech, Marseilles, France); mouse anti-human CD49a monoclonal antibody (clone TS2/7, Serotec Ltd., Oxford, UK); mouse anti-human CD34 monoclonal IgG (clone Bl-3C5, Zymed Lab., San Francisco, CA, USA); mouse anti-human CD49b monoclonal

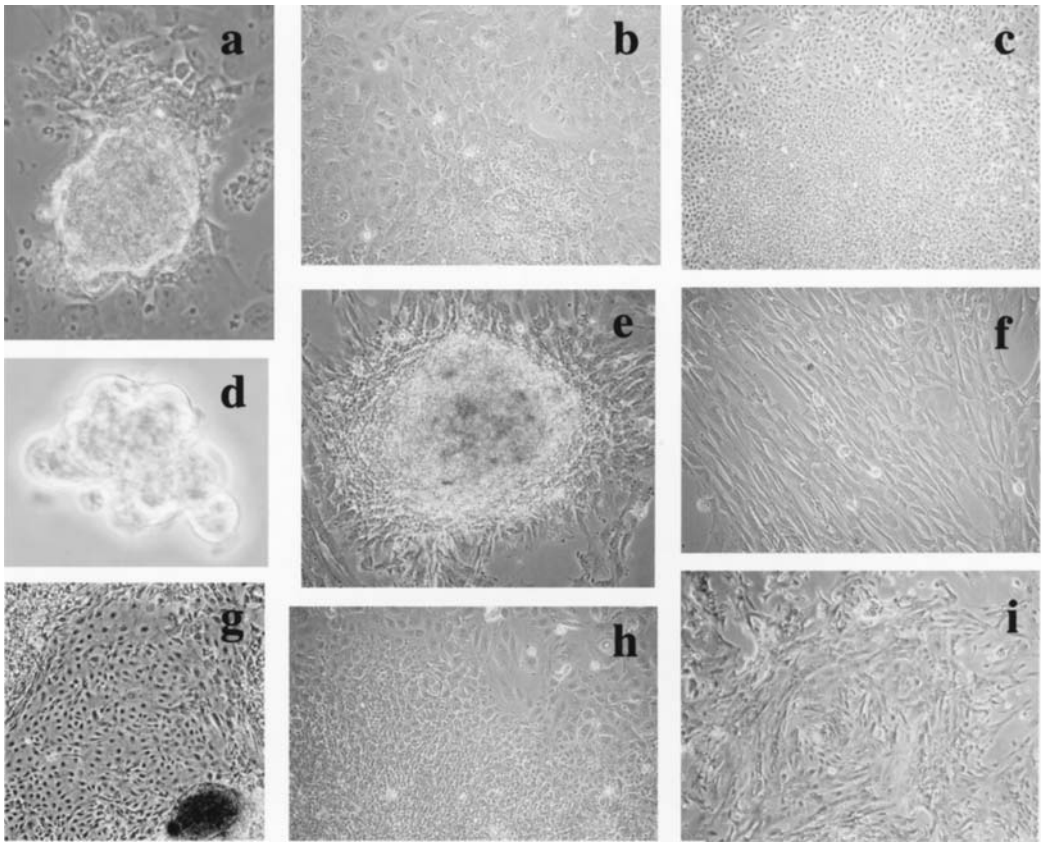

Figure 3. Morphology of the cultured primary cells from a surgically-excised ovarian tumor. (a-i) The primary tumor cells showed a varying morphology *in vitro*. They grew well *in vitro* and formed colonies together with cells of the same morphology. (d) The non-adherent cells that grew slowly, forming floating colonies, are shown.

antibody (clone 31H4, Serotec Ltd.); mouse anti-human CD49c monoclonal antibody (clone 11G5, Cymbus Biotech Ltd.); mouse anti-human CD49d monoclonal antibody (clone 44H6, Cymbus Biotech Ltd.); mouse anti-human CD49e monoclonal antibody (clone SAM1, Beckman Coulter Japan); mouse anti-human CD49f monoclonal antibody (clone 4F10, Cymbus Biotech Ltd.); mouse anti-human CD40 monoclonal antibody (clone MAB89, Beckman Coulter Japan); mouse anti-human Fas (CD95) monoclonal IgG (clone UB2, MBL, Nagoya, Japan) and mouse anti-human TNFR1p60 monoclonal antibody (Genzyme).

Flow cytometric analyses of intracellular antigens. Flow cytometric analyses of intracellular antigens such as cytokeratin, smooth muscle actin (SMA), vimentin and the von Willebrand factor (vWF) were performed. Cells were detached from the culture flask with 3 mM EDTA in phosphate-buffered saline (PBS) and washed with PBS/2% FCS. Cells were fixed with 4% paraformaldehyde in 0.1 M NaH_2PO_4 at pH 7.4 for 10 min on ice. After the cells were washed twice with wash buffer (PBS/2% FCS), they were treated with 100 μg/ml digitonin (Sigma) for 10 min at RT and washed twice again. Cells were treated with normal mouse IgG to block non-specific binding sites for 5 min at RT followed by incubation in one of the monoclonal anti-human antigens for 30 min at RT and two further washes. Finally, the cells were incubated in FITC-conjugated goat anti-mouse IgG (Gibco BRL) for 30 min at RT, washed twice and suspended in 200 μl of wash buffer for analyses by FACSCalibur (Beckman Coulter Japan). The primary antibodies used in these analyses were: mouse anti-cytokeratin antibody (clone AE1/AE3, Progen); mouse anti-vimentin monoclonal antibody (Dako Japan); mouse anti-SMA monoclonal antibody (clone 1A4, Neomarkers, Fremont, CA, USA) and mouse anti-vWF monoclonal antibody (clone 4F9, Immunotech).

Results

Primary cell culture. Primary culture cells dissociated from the resected ovarian tumor proliferated well in the estrogen-containing medium. Primary cells grown *in vitro* were morphologically variable in shape and growth mode, in corroboration with the aforementioned histological findings (Fig. 3a-i). Morphologically similar cells aggregated together, whereas morphologically different cells did not grow together, even in mixed cultures. When passaged, the number of non-adherent floating tumor cells decreased, but adherent varieties proliferated independently for seven months without any morphological changes.

Effects of cytokines and growth factors on the morphology of primary cells. It is reported that the proliferation and differentiation of immature teratoma cells can be inhibited or stimulated by certain cytokines and growth factors (21,26,27).

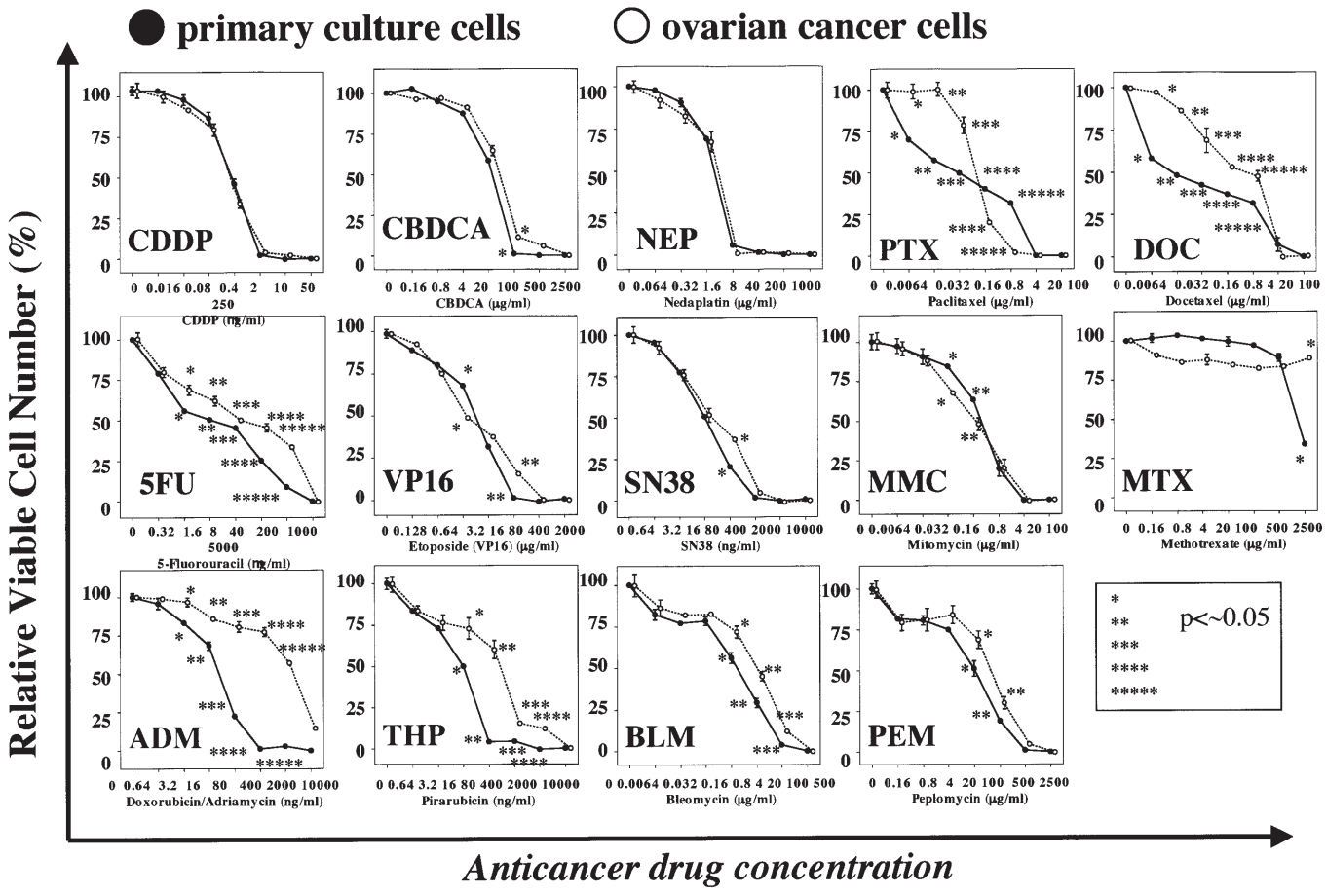

Figure 4. Comparison of the anticancer drug-sensitivity of primary cells derived from an ovarian immature teratoma and those derived from the ascites of an ovarian cancer patient with carcinomatous peritonitis. The solid lines with the black circles show the drug-sensitivity curves of the primary cultured teratoma cells. The dotted lines with the white circles show the drug-sensitivity curves of the peritoneal cancer cells.

Therefore, we tested the effects of 25 cytokines and growth factors on primary cell cultures. The cells were observed daily, but there were no changes in cell proliferation or morphology during the three-week period of observation (data not shown).

Anticancer drug-sensitivity of primary cultured cells. The anticancer drug sensitivity of primary cultured cells was compared to that of the *in vitro*-cultured peritoneal adenocarcinoma cells obtained from the ascitic fluid of a patient with ovarian adenocarcinomatous peritonitis, who was treated at the same time as the immature teratoma patients (Fig. 4). There was no significant difference in the sensitivity to three platinum drugs (CDDP, CBDCA and NPL) between the immature teratoma and the peritoneal cancer cells. However, the immature teratoma cells were more sensitive to taxane compounds such as PTX and DOC, highly sensitive to ADM and THP but moderately sensitive to 5FU, BLM and PEM. Sensitivity to VP16, SN38 and MMC was only slightly higher in the teratoma cells than in the peritoneal cancer cells.

Nude mouse transplantation. To generate an experimental model of recurrent ovarian immature teratoma, we transplanted nude mice with the primary teratoma cells. A variety of cultured primary cells were mixed and ~10^8 cells were subcutaneously transplanted into the backs of nude mice. The transplant masses had almost disappeared within one month, and then irregular tumors grew. Three months later, the tumors contained osteoid hard tissues (Fig. 5a and b). A histopathological examination of the nude mouse tumors showed heterogeneous tissue containing nerve, osteoid and cartilage-like tissues similar to the primary tumor histology (Fig. 5d and e). The nude mouse tumor was resected and dissociated to generate further cell cultures. Nude mouse tumor cells (~10^8) were transplanted again into three more nude mice. The transplanted mass disappeared in less than one month but four months later a tumoral lesion appeared.

Karyotyping analysis. Immature teratoma cells with chromosomal aberrations are reportedly highly malignant in character (28,29). Therefore a chromosomal analysis of the cells cultured from the nude mouse tumor was performed. All 20 cells examined showed a normal 46XX karyotype (Fig. 5c).

Limiting dilution analysis. If heterogeneous immature teratoma cells are derived from a few stem cell-like tumor cells, tumor colonies can be detected in the limiting dilution cultures. Based on this hypothesis, we attempted limiting the dilution cultures of tumor cells derived from primary cell cultures two times. Colonies had not been established, even in wells with 30 cells/well, four weeks after the limiting

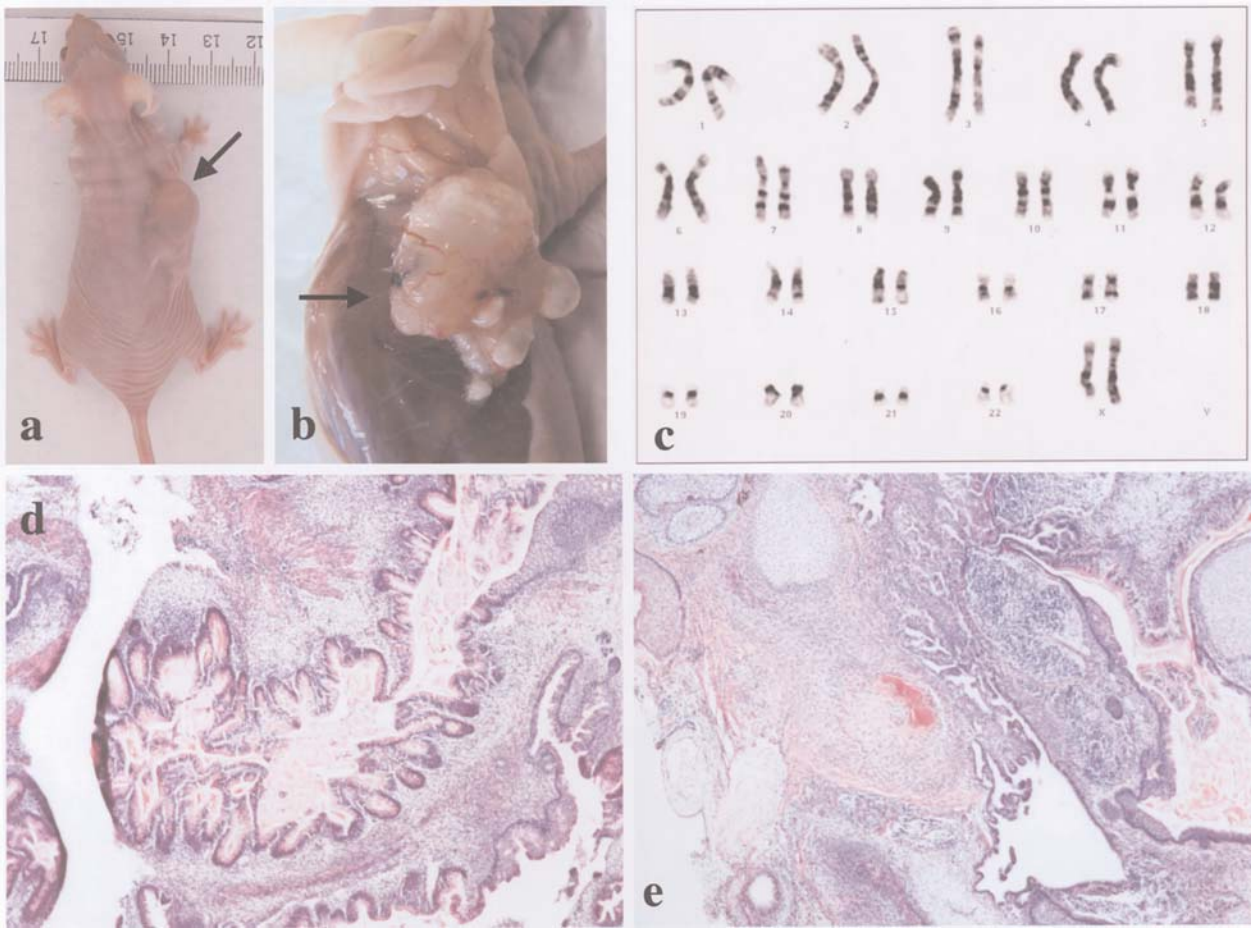

Figure 5. Nude mouse transplantation experiment. (a and b) A tumor-bearing nude mouse. (c) Karyotyping of the nude mouse tumor cells. The nude mouse tumor cells were grown *in vitro* and karyotyped. All 20 of the cells examined had a normal 46XX karyotype. (d and e) Histopathological findings from the nude mouse tumor tissue.

dilution. Similarly, we failed to establish monoclonal cell lines from two attempts at limiting the dilution culture of cells grown *in vitro* from the nude mouse tumor.

Anticancer drug-sensitivity of nude mouse tumor cells. The anticancer drug sensitivity of nude mouse tumor cells was compared to that of primary cultured cells (Fig. 6). Nude mouse tumor cells were less sensitive to 12 out of the 14 anti-cancer drugs examined. In particular, the sensitivity of nude mouse tumor cells to PTX, DOC, ADM, THP and BLM decreased greatly. However, nude mouse tumor cells were equally sensitive to CBDCA and PEM as the primary cultured cells.

Flow cytometry. Tumors that recur after optimal surgery are thought to be composed of selected cells that can reconstitute *in vivo* when transplanted into nude mice. Therefore, we examined whether there was any difference in the expression of antigens, such as cell lineage antigens or integrins between the primary tumor cells and the nude mouse tumor cells by flow cytometric analyses. As shown in Fig. 7, out of the 16 antigens examined, SMA expression was the only differentially expressed antigen. The SMA expression in the nude mouse tumor cells also significantly decreased when compared to that of the primary cultured cells.

Discussion

Since ovarian immature teratoma occurs frequently in young women, optimal minimal surgery, usually unilateral oophorectomy, is performed with the exception of patients with advanced disease or patients who do not hope for a future pregnancy (1-3). However, chemotherapy is a necessity if the tumor recurs after optimal surgery but there have been no experimental studies to determine which anticancer drugs are most suitable for such patients.

Experimental observations of cultured ovarian immature teratoma cells led to several noteworthy results. Primary cells derived from the ovarian immature teratoma were morphologically diverse, a finding that is consistent with primary tumor histopathology. However, when morphologically different primary cells were cultured together, mixed colonies did not form. Moreover, we added 25 different cytokines or growth factors to cultures containing primary cells of varying morphology, but observed no changes in cell morphology or the rate of proliferation. These results suggest that the tumorigenesis of immature teratoma cells requires a mixture of several cell types that are stable enough to proliferate, possibly in an autocrine fashion, without differentiating for long periods. These cells are also stable enough not to

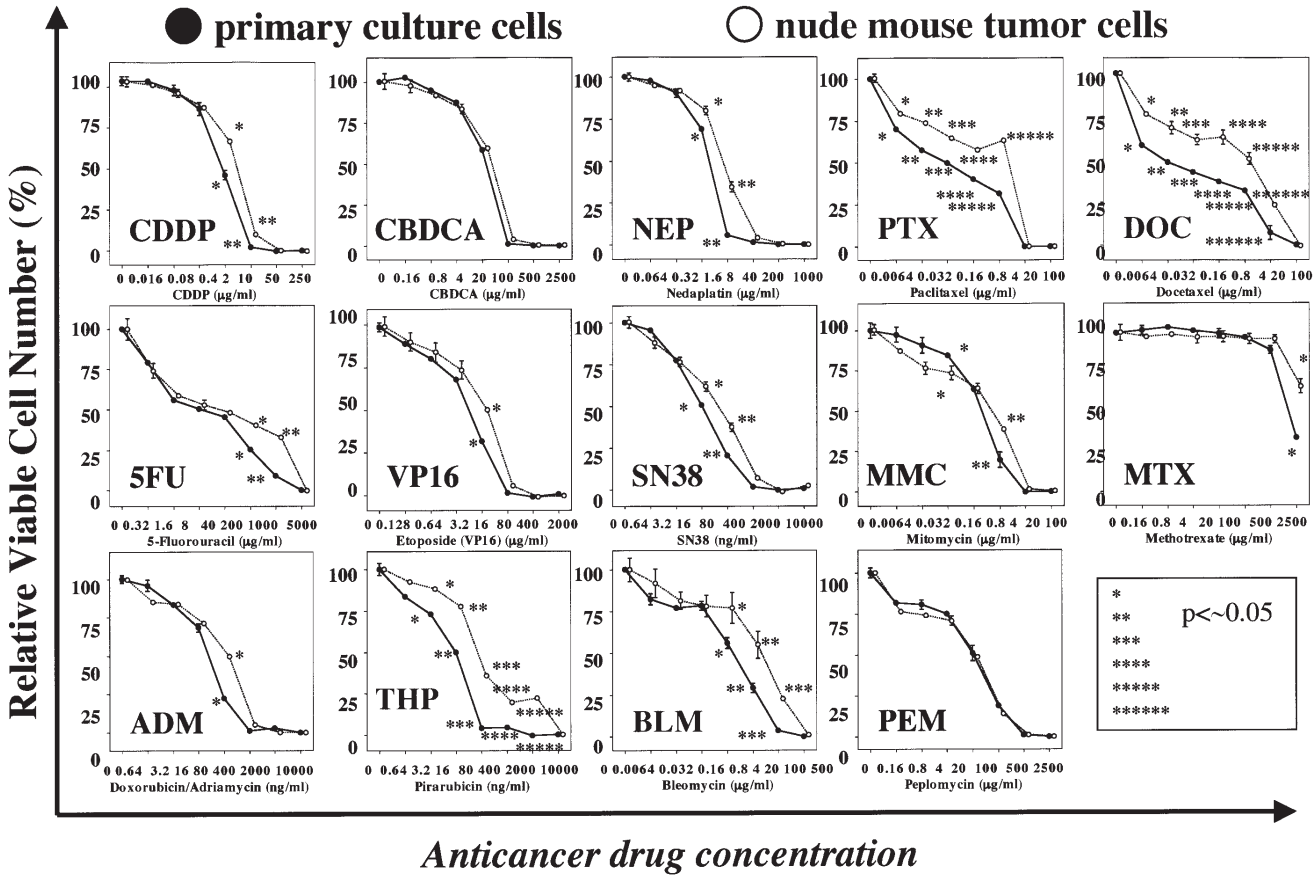

Figure 6. Comparison between the anticancer drug sensitivity of primary cultured cells and nude mouse tumor cells. The solid lines with the black circles show the drug-sensitivity curves of the primary cultured teratoma cells. The dotted lines with the white circles show the drug-sensitivity curves of the nude mouse tumor cells.

differentiate and construct a functional organ-like structure. The stability of these tumor cells may mean that this tumor does not readily transform.

Cells from the nude mouse transplantation experiments and the *in vitro* culture experiments demonstrated some characteristics of clinically recurrent ovarian immature teratoma cells after optimal surgery. The limiting dilution culture experiments suggest that for an immature teratoma tumor to recur after minimal optimal surgery, there needs to be >30 tumor cells left in the body after the first surgery. Moreover, to regenerate the heterogeneous tumor tissues that recur after surgery, several types of tumor cells are needed. The present experiments demonstrate that recurrent ovarian immature teratoma tumors may be formed by a mixture of many different cells, not just from a few remaining stem cell-like cancer cells, as found with more common cancers. This may explain why patients with ovarian immature teratomas have a better prognosis.

There have been few reports regarding the chromosomal analysis of ovarian immature teratoma cells (28-33). The majority of immature teratoma cells are reported to have an abnormal karyotype. Ovarian immature teratoma cells with abnormal karyotypes have a higher incidence in recurring tumors and metastases and these are clinically evaluated as malignant tumors. In contrast, all available reports of ovarian immature teratoma with a normal 46XX karyotype state that tumors did not recur after surgery (28,29). The teratoma cells established in culture in this study had a normal 46XX karyotype but were capable of forming tumors in the nude mice. Therefore, ovarian immature teratoma cells with a normal karyotype must have a malignant potential. However, the primary cultures of teratoma cells established in this study were stable enough not to change their rate of proliferation or morphology in the presence of 25 different bioactive stimuli. Moreover, the patient from which the cells were derived has not had a relapse in the 57+ months since surgery, suggesting that primary tumors with a normal karyotype must have a very low malignant potential.

The anticancer drug sensitivity of primary cultured teratoma cells was compared to that of cultured cells derived from the peritoneal cancer cells of a patient with adenocarcinomatous peritonitis. As for the sensitivity to three platinum drugs (CDDP, CBDCA and NPL), there was little difference in sensitivity between the immature teratoma and the ovarian adenocarcinoma cells. However, with regard to sensitivity to taxane compounds such as PTX and DOC, immature teratoma cells were more highly drug-sensitive than the adenocarcinoma cells. The immature teratoma cells were extremely sensitive to ADM and THP, and moderately more sensitive to 5FU, BLM and PEM when compared to the adenocarcinoma cells. Teratoma cell sensitivity to VP16, SN38 and MMC was very slightly higher than that of the adenocarcinoma cells. However, it cannot be said that the anticancer drug sensitivity of the peritoneal adenocarcinoma

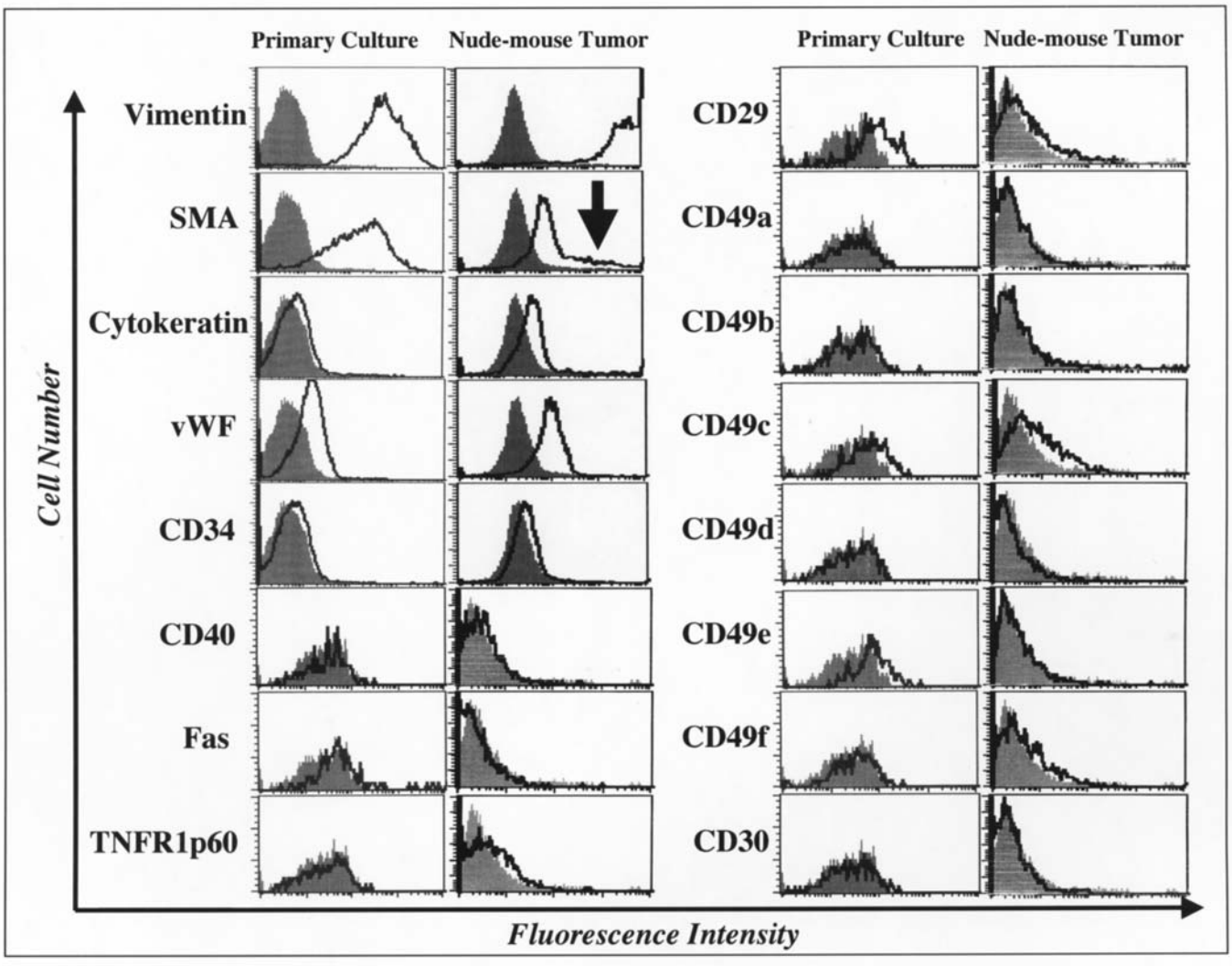

Figure 7. Flow cytometric analyses of primary and nude mouse tumor cells. The differential expression of 16 cell surface antigens was investigated by simultaneous flow cytometry with excessive antibody exposure. SMA expression was apparently lower in the nude mouse tumor cells than in the primary tumor cells. However, the expression of the remaining 15 antigens was similar between the two cell types.

cells examined here is representative of all ovarian cancer cells.

The anticancer drug sensitivity of malignant ovarian germ-cell tumors is usually high and PVB (11,12) and BEP (13,14) are the most frequently administered drug combinations for patients with ovarian immature teratomas all over the world. However, there have been no reports to examine whether PVB or BEP regimens are the best anticancer chemotherapeutic protocols for immature teratoma patients. In this study, the primary culture cells derived from the ovarian immature teratoma showed a significantly higher sensitivity for the main constituent of anticancer drugs in VAC, PVB, and BEP regimens than the peritoneal cancer cells, thereby providing experimental proof that BEP and PVB are adequate anticancer drug regimens for malignant ovarian germ-cell tumors. However, particularly strong ovarian toxicity is reported for cyclophosphamide (15-17), etoposide (VP-16) (18-20) and irinotecan-HCl (a prodrug of SN38) (34-36) and use of these drugs may induce premature ovarian failure. There is a need for more efficacious anticancer drugs with a lower ovarian toxicity for the treatment of patients with a hope of future pregnancy, since most patients with ovarian immature teratoma are young and want to avoid the gonadal toxicity of chemotherapy. Since anticancer drug sensitivity of teratoma cells for taxane compounds such as PTX and DOC were very high, paclitaxel/carboplatin or docetaxel/carboplatin, which are frequently administered for epithelial ovarian cancers, may be given as an alternative first-line of chemotherapy for nulliparous young patients with ovarian immature teratomas.

For the first time, we report a change in the anticancer drug sensitivity of the ovarian immature teratoma cells before and after nude mouse transplantation. The present results may be of use when choosing an anticancer drug regimen to treat a recurring ovarian immature teratoma after optimal surgery. Nude mouse tumor cell drug-sensitivity decreased when compared to the primary teratoma cells, while the sensitivity to CBDCA and PEM remained unchanged. Specifically, the sensitivity of nude mouse tumor cells to PTX, DOC, ADM, THP and BLM greatly decreased. Therefore, it was supposed that primary tumor cells with a lower anticancer drug sensitivity were more likely to survive transplan-

tation. The nude mouse tumor of ovarian immature teratoma cells closely resembled the clinical form of recurrent immature teratoma after optimal surgery. Therefore, it is highly possible that clinical ovarian immature teratomas that recur after minimal operations are less sensitive to anticancer drugs than the primary tumor cells, even though the patients did not undergo chemotherapy. These results may suggest that postoperative adjuvant chemotherapy in the case of a patient who does not hope for a future pregnancy, may kill the sensitive residual tumor cells radically. Furthermore, it is very likely that adjuvant chemotherapy can lower the postoperative recurrence rate because our results suggest that tumor colony formation requires >30 residual tumor cells, as shown by limiting dilution analyses.

The antigenicity of nude mouse tumor cells was compared with primary teratoma cells by flow cytometry to clarify which tumor cell types selectively survived transplantation. Of the 14 antigens tested, the only alteration was an apparent decrease in SMA expression in the nude mouse tumor cells. These results indicate that transplantation led to the selective exclusion of SMA-overexpressing cells from the primary tumor cells, and that SMA-overexpressing cells may be more drug-sensitive.

The present investigation has resulted in the establishment of a novel cell line derived from human ovarian immature teratoma, which could be applied for *in vitro* and/or *in vivo* studies. The specific properties of these ovarian immature tumor cells are described for the first time and will provide important information with regard to the pathogenesis and future therapies of ovarian immature teratoma. In addition, our results suggest that postoperative adjuvant chemotherapy can lower the risk of ovarian immature teratomas recurring.

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