monocytes was up-regulated by IFN-γ or a soluble fusion protein of CTLA-4 and immunoglobulin Fc (CTLA-4/Fc) treatment during normal pregnancy, but both IDO expression on DC and monocytes with IFN-γ or CTLA-4/Fc treatment were decreased in spontaneous abortion cases, suggesting that $CD4^+CD25^+$ Treg cells and IFN-γ play important roles in the maintenance of pregnancy by the up-regulation of IDO expression.

Materials and methods

Blood and tissue samples

Heparinized venous blood samples and decidual samples were obtained from induced abortion cases (n = 14; age 30.2 ± 4.5 years; gestational age at sampling 7.0 ± 1.3 weeks) and spontaneous abortion cases (n = 10) in the first trimester (age 31.5 ± 4.1 years; gestational age at sampling 7.2 ± 1.8 weeks). We obtained decidual samples and peripheral blood samples from the same patients. Blood samples were obtained from non-pregnant healthy women (n = 10; age 30.5 ± 3.1 years). Informed consent was obtained from all patients. Peripheral blood mononuclear cells were isolated by the standard Ficoll–Hypaque method. The decidual tissues were macroscopically separated from chorionic villi and then cut into small pieces using a razor blade and vigorously shaken for 2 min. These samples were then filtered through a 32 μm nylon mesh, and decidual mononuclear cells (leucocytes) were purified by the Ficoll–Hypaque method (Saito *et al.*, 1992; Tsuda *et al.*, 2002). Decidual tissues were not enzymatically digested to prevent enzymatic treatment from affecting the fluorescence intensity of surface antigens. The yield of decidual leukocytes was 8.5 ± 3.0 × 10^6 cells. All of the sampling and use of tissues for this study were approved by the Toyama Medical and Pharmaceutical University Ethics Committees.

Culture system and IDO expression by flow cytometry

Isolated mononuclear cells (1 × 10^6/ml) were cultured using Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Tokyo, Japan), supplemented with 10% heat-inactivated fetal calf serum in 12 well flat-bottomed plates for 24 h in the presence or absence of IFN-γ (R&D Systems, Minneapolis, MN, USA) or recombinant human CTLA-4/IgG Fc chimera (CTLA-4/Fc) (R&D Systems). For flow cytometric analysis, non-adherent and adherent cells were collected with a cell scraper after 24 h. Cells were first stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD14 mouse monoclonal antibody (mAb) (Becton Dickinson, San Diego, CA, USA) or FITC-conjugated lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56; Becton Dickinson) mAbs and PE-conjugated anti-HLA-DR mAb (Becton Dickinson). Then, to stain intracellular molecules, cells were treated with permeabilizing solution (Becton Dickinson). These cells were secondarily stained with a biotin-labelled anti-human IDO mAb (Takikawa *et al.*, 1988), followed by streptavidin-PC5 (Beckman Coulter, Marseille, France). Isotype-matched mouse IgGs were used as a negative control. Flow cytometric analysis was performed on a fluorescence-activated cell sorter (FACS) Calibur using CellQuest software (Becton Dickinson). To analyse macrophages, a gate was set around the monocytes based on forward and side scatter (Figure 1, upper left) and then a gate was set on $CD14^+$ cells (Figure 1, upper middle). After that, the population of intracellular IDO^+ cells in $CD14^+$ cells was calculated (Figure 1, upper right). To analyse DCs, a gate was set around the lymphocytes avoiding granulocytes (Figure 1, lower left) and a gate was set on lineage $markers^-$/$HLA\text{-}DR^+$ (Figure 1, lower middle). After that, the population of intracellular IDO^+ cells in lineage $markers^-$/$HLA\text{-}DR^+$ cells was calculated (Figure 1, lower right).

Cytokine quantitation

Culture supernatants were collected and analysed by the enzyme-linked immunosorbent assay (ELISA) method. An ELISA kit (ENDOGEN, Rockford, IL, USA) was used to quantify human IFN-γ in supernatants.

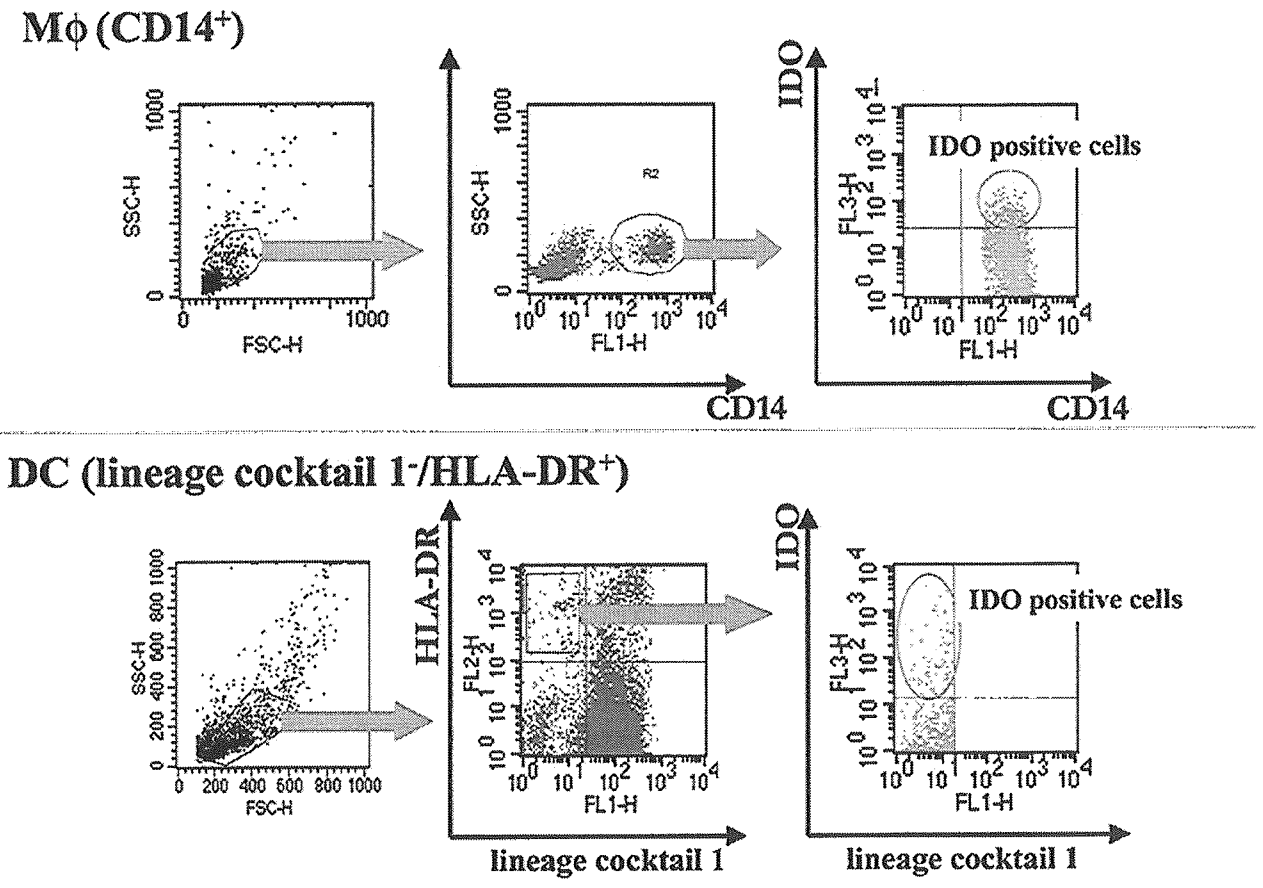

Figure 1. Indoleamine 2,3-dioxygenase (IDO)-positive cells in $CD14^+$ monocytes and lineage $markers^-$/$HLA\text{-}DR^+$ dendritic cells (DCs) analysed by flow cytometry.

B7 molecule expression by flow cytometry

Isolated mononuclear cells were stained with FITC-conjugated anti-CD14 mAb or FITC-conjugated lineage cocktail mAbs and APC-conjugated anti-HLA-DR mAb (Becton Dickinson). Then, to stain B7 molecules, cells were also stained with a PE-conjugated anti-CD80 mAb (Becton Dickinson) and biotin-labelled anti-CD86 mAb (Becton Dickinson), followed by streptavidin-PC5.

Statistical analysis

The data were analysed by Friedmann test for paired samples or Mann–Whitney *U*-test for unpaired samples. $P < 0.05$ was considered significant.

Results

IDO expression in peripheral blood and decidual monocytes and DCs

First, we examined the dose dependency of IFN-γ or CTLA-4/Fc in the expression of IDO on peripheral blood monocytes and DCs of normal early pregnant women ($n = 3$). Treatment with IFN-γ or CTLA-4/Fc both increased IDO expression dose dependently and IDO expression reached a plateau at 1000 U/ml of IFN-γ and 10 μg/ml of CTLA-4/Fc (Figure 2). From these results, we decided to use 1000 U/ml of IFN-γ and 10 μg/ml of CTLA-4/Fc.

As shown in Figure 3, under non-stimulated conditions, the expression of IDO in peripheral blood monocytes and DCs of non-pregnant subjects was very low. The expression rates in both peripheral blood monocytes and DCs of normal pregnancy subjects were significantly higher ($P = 0.0077$ and $P = 0.0077$, respectively) than those of non-pregnant subjects. Both IFN-γ and CTLA-4/Fc treatment enhanced the population of IDO-expressing peripheral blood monocytes and DCs of non-pregnant subjects. IFN-γ induced higher IDO expression in monocytes compared with that by CTLA-4/Fc, and CTLA-4/Fc induced higher IDO expression in DCs compared with that by IFN-γ. In spontaneous abortion cases, IDO expression of monocytes with IFN-γ treatment was significantly lower ($P = 0.0139$) compared with that in normal pregnancy subjects. CTLA-4/Fc-induced IDO expression in peripheral blood DCs of spontaneous abortion cases was significantly lower ($P = 0.0192$) than that in normal pregnancy subjects.

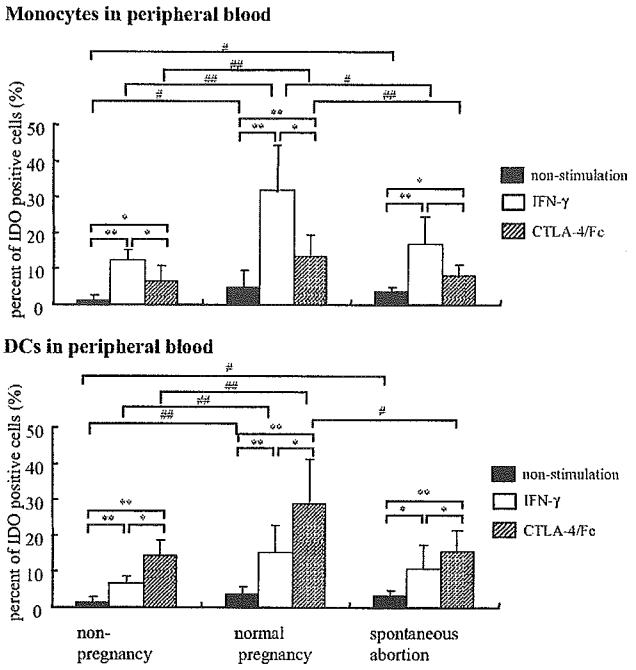

Figure 3. Comparison of indoleamine 2,3-dioxygenase (IDO) expression between normal pregnancy and spontaneous abortion in peripheral blood monocytes and dendritic cells (DCs). *, $P < 0.05$ and **, $P < 0.01$, respectively, when the data were analysed by Friedmann test. #, $P < 0.05$ and ##, $P < 0.01$, respectively, when the data were analysed by Mann–Whitney *U*-test.

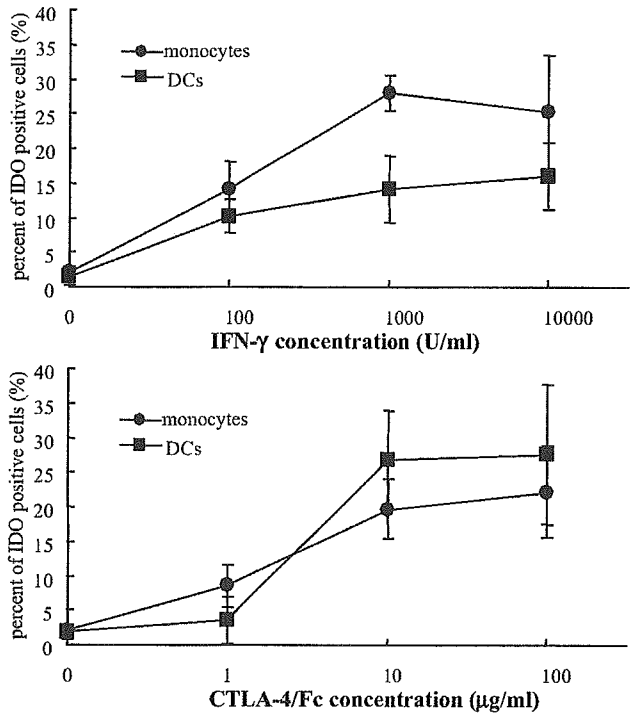

Figure 2. Indoleamine 2,3-dioxygenase (IDO) expression in monocytes and dendritic cells (DCs) stimulated by a range of doses of interferon gamma (IFN-γ) or CTLA-4/Fc. Peripheral blood mononuclear cells were treated for 24 h with IFN-γ (at concentrations of 0, 100, 1000 and 10 000 U/ml) or CTLA-4/Fc (at concentrations of 0, 1, 10 and 100 μg/ml). The percent of IDO-positive cells of monocytes or DCs were analysed by flow cytometry.

IDO expression in non-stimulated decidual monocytes and DCs of spontaneous abortion cases were significantly lower ($P = 0.0002$ and $P = 0.0029$, respectively) than those in normal pregnant women (Figure 4). IDO expression in both decidual monocytes and DCs were significantly elevated by both IFN-γ and CTLA-4/Fc treatments. IFN-γ mainly augmented the expression of IDO in monocytes, whereas CTLA-4/Fc mainly augmented the expression in DCs. The response levels of decidual monocytes in spontaneous abortion cases with both IFN-γ and CTLA-4/Fc treatments were significantly lower ($P = 0.0028$ and $P = 0.010$, respectively) compared with those in normal pregnant subjects. The response levels of decidual DCs in spontaneous abortion cases by CTLA-4/Fc treatment were significantly lower ($P = 0.0032$) compared with those in normal pregnant subjects.

IFN-γ production induced by CTLA-4/Fc

It has been reported that CTLA-4 induces IDO expression in DC through the induction of IFN-γ production (Grohmann *et al.*, 2002; Munn *et al.*, 2004). To investigate whether IDO expression by CTLA-4/Fc was through IFN-γ production, we checked the IFN-γ secretion by mononuclear cells treated by CTLA-4/Fc for 24 h.

As shown in Figures 5 and 6, CTLA-4/Fc induced IFN-γ secretion by both peripheral blood mononuclear cells and decidual leucocytes.

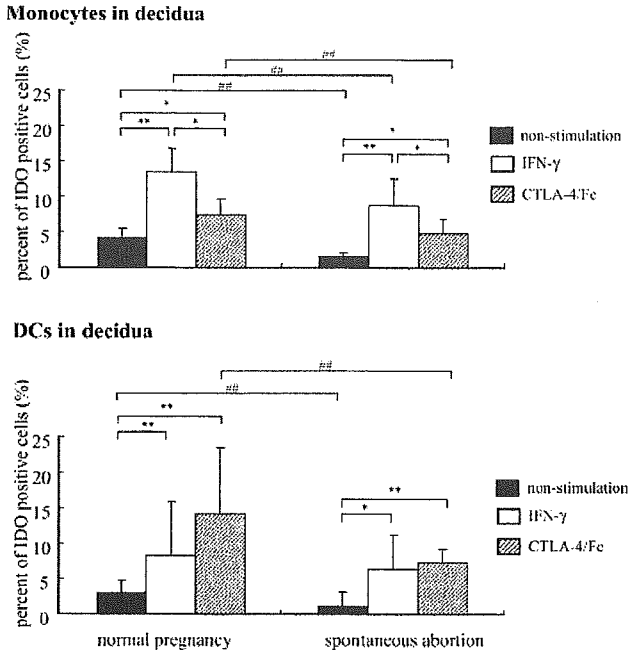

Figure 4. Comparison of indoleamine 2,3-dioxygenase (IDO) expression between normal pregnancy and spontaneous abortion in decidual monocytes and dendritic cells (DCs). *, $P < 0.05$ and **, $P < 0.01$, respectively, when the data were analysed by Friedmann test. #, $P < 0.05$ and ##, $P < 0.01$, respectively, when the data were analysed by Mann–Whitney *U*-test.

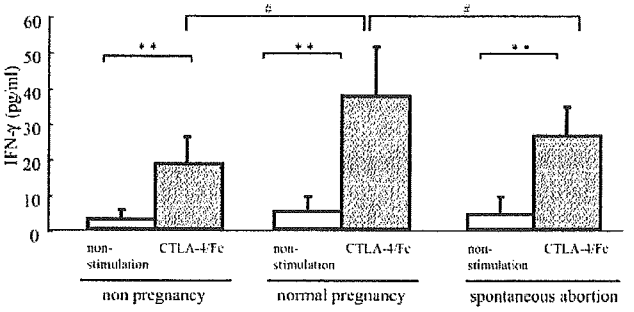

Figure 5. Interferon gamma (IFN-γ) concentration in the supernatant of peripheral blood mononuclear cells stimulated by CTLA-4/Fc. **, $P < 0.01$ when the data were analysed by Friedmann test. #, $P < 0.05$ when the data were analysed by Mann–Whitney *U*-test.

CTLA-4/Fc-induced IFN-γ secretion by peripheral blood mononuclear cells in normal pregnancy subjects was significantly higher compared with those in non-pregnancy subjects (37.8 ± 13.8 pg/ml versus 18.0 ± 7.6 pg/ml, $P = 0.0126$). IFN-γ levels in spontaneous abortion cases were significantly lower compared with those in normal pregnancy subjects in both peripheral blood and decidua (26.0 ± 8.2 pg/ml versus 37.8 ± 13.8 pg/ml; $P = 0.048$ and 307.9 ± 237.6 versus 663.9 ± 277.4; $P = 0.0052$, respectively).

The expression of B7 molecules in peripheral blood and decidual monocytes and DCs

It has been reported that engagement of B7-1/B7-2 (CD80/CD86) molecules on monocytes and DCs by CTLA-4/Fc activates a signalling pathway leading to the induction of IDO (Fallarino *et al.*, 2003; Munn *et al.*, 2004). We investigated whether the expression of IDO correlated with the expression of B7 molecules in peripheral and decidual monocytes and DCs.

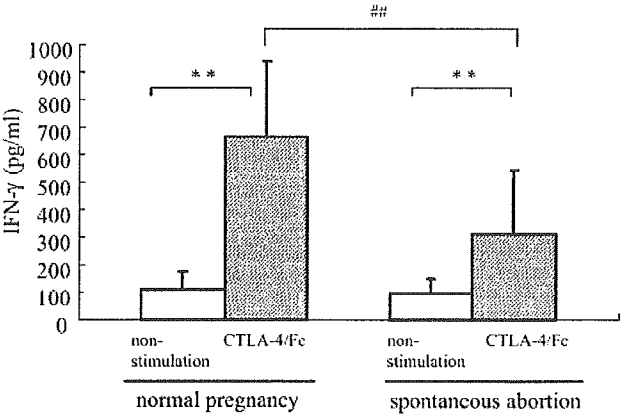

Figure 6. Interferon gamma (IFN-γ) concentration in the supernatant of decidual leucoctyes stimulated by CTLA-4/Fc. **, $P < 0.01$ when the data were analysed by Friedmann test. ##, $P < 0.01$ when the data were analysed by Mann–Whitney *U*-test.

As summarized in Table I, CD86 expression on peripheral blood monocytes and DCs in normal pregnancy subjects was significantly higher than those in non-pregnant subjects. CD86 expressions on peripheral blood monocytes, decidual monocytes and decidual DCs in spontaneous abortion cases were significantly lower compared with those in normal pregnancy subjects. On the other hand, CD80 expression on both peripheral blood and decidual monocytes and DCs in normal pregnancy subjects was the same as those in spontaneous abortion cases.

Discussion

During pregnancy, the fetus is prevented from maternal immune rejection. Several specialized mechanisms have evolved to protect the semi-allograftic fetus from maternal immune attack. Studies in mice have suggested that the fetus is protected from immune rejection by maternal T cells by means of IDO-dependent depletion of tryptophan (Munn *et al.*, 1998). Recent data suggest that $CD4^+CD25^+$ Treg cells expand during pregnancy (Aluvihare *et al.*, 2004; Heikkinen *et al.*, 2004; Sasaki *et al.*, 2004; Somerset *et al.*, 2004; Zenclussen *et al.*, 2005), and Treg cells mediate maternal tolerance to the fetus (Aluvihare *et al.*, 2004). Treg cells constitutively express CTLA-4, and recently it was proposed that CTLA-4 induces IFN-γ production by DCs, which leads to the expression of IDO in DCs (Fallarino *et al.*, 2003; Munn *et al.*, 2004). Thus, the two mechanisms that induce maternal tolerance to the fetus have been linked.

We studied IDO expression on decidual and peripheral blood DCs and monocytes by CTLA-4/Fc using flow cytometry. CTLA-4/Fc treatment induced IDO expression in both decidual and peripheral blood DC and monocytes. The amplitude of IDO expression in DCs by CTLA-4/Fc was rather high compared with monocytes. On the other hand, the amplitude of IDO expression by IFN-γ in monocytes was rather high compared with DCs. Heikkinen *et al.* (2004) reported that IFN-γ induced the expression of IDO mRNA in peripheral blood $CD14^+$ monocytes from pregnant women, but CTLA-4/Fc did not induce IDO mRNA in peripheral blood monocytes. Their data are inconsistent with our data. Our data showed that CTLA-4/Fc induced IDO expression in both peripheral blood and decidual $CD14^+$ monocytes, although the expression rate of IDO was rather low compared to

Table I. Comparison of the expression of B7 molecules between normal pregnancy and spontaneous abortion

	Monocytes [Mean ± SD (%)]			Dendritic cells [Mean ± SD (%)]		
	Non-pregnancy (n = 10)	Normal pregnancy (n = 10)	Spontaneous abortion (n = 8)	Non-pregnancy (n = 10)	Normal pregnancy (n = 10)	Spontaneous abortion (n = 8)
Peripheral blood						
CD80	2.35 ± 2.32	3.72 ± 4.30	4.11 ± 5.10	5.67 ± 1.83	5.38 ± 2.25	3.64 ± 3.36
CD86	33.40 ± 12.88*	60.75 ± 26.54	35.45 ± 14.08*	8.55 ± 2.23*	14.15 ± 5.70	10.80 ± 8.70
Decidua						
CD80	Not tested	7.06 ± 5.81	7.17 ± 6.03	Not tested	4.14 ± 1.68	5.26 ± 2.42
CD86	Not tested	54.29 ± 21.88	19.07 ± 9.60†	Not tested	17.83 ± 4.92	8.20 ± 4.72†

The data were analysed by Mann–Whitney U-test.
*$P < 0.05$ versus normal pregnancy.
†$P < 0.01$ versus normal pregnancy.

that with IFN-γ treatment. Post-transcriptional regulation of IDO may induce IDO protein expression in monocytes with CTLA-4/Fc treatment.

Binding of CTLA-4 and CD80/86 (B7 complex), followed by IFN-γ production by DCs, is required for the induction of IDO by Treg cells (Fallarino *et al.*, 2003; Munn *et al.*, 2004). These data showed that CD86 expression on peripheral blood monocytes and DCs and decidual DCs of spontaneous abortion cases was significantly decreased compared with normal pregnant subjects. These findings might explain why the CTLA-4/Fc-mediated IFN-γ secretion by decidual mononuclear cells in spontaneous abortion cases was significantly lower than that in normal pregnancy subjects (Figure 6). To clarify the mechanism of IDO expression in DCs and monocytes by CTLA-4/Fc, we examined the IFN-γ secretion level by CTLA-4/Fc-stimulated peripheral blood mononuclear cells and decidual mononuclear cells. The results showed that IFN-γ production by peripheral and decidual mononuclear cells after CTLA-4/Fc treatment in spontaneous abortion cases was significantly lower than those in normal pregnancy subjects, suggesting that IFN-γ production by the binding of CTLA-4 on Treg cells and CD80/86 expressing DC and monocytes could induce IDO expression in normal pregnancy subjects. However, in spontaneous abortion cases, suppressed IFN-γ production by the binding of CTLA-4 and decreased CD80/86 expressing DC and monocytes might reduce the IDO expression in DC and monocytes. It is well known that cytokine profiles in decidual T cells are in the Th2-dominant state (Piccinni *et al.*, 1998; Saito *et al.*, 1998; Tsuda *et al.*, 2002; Kwak-Kim *et al.*, 2003; Michimata *et al.*, 2003), so the finding that CTLA-4/Fc treatment induces a large amount of IFN-γ secretion by decidual mononuclear cells is interesting. Decidual mononuclear cells contain IFN-γ-producing NK cells, monocytes, T cells, NKT cells and DCs. Further study is needed to clarify which cells in the decidua produce IFN-γ after CTLA-4/Fc treatment.

Another important finding is that IDO expression in unstimulated decidual DCs and monocytes was up-regulated compared with those in peripheral blood. Human pregnancy decidua contains an abundance of Treg cells which express CTLA-4 at a high level (Heikkinen *et al.*, 2004; Sasaki *et al.*, 2004). These findings suggest that surface CTLA-4 on Treg in the decidua induces IDO expression in decidual DCs and monocytes preventing the maternal lymphocyte activation against the fetal allograft. It has been reported that the population of decidual $CD4^+CD25^+$ Treg cells and the surface CTLA expression on Treg cells are significantly lower in decidua from spontaneous abortion compared with those from specimens from induced abortion (Sasaki *et al.*, 2004). In this study, IDO expressions in both decidual DCs and monocytes of spontaneous abortion cases were suppressed compared with those of normal pregnant subjects. Decreased CTLA-4-expressing Treg cells and suppressed IFN-γ production by CTLA-4 in the decidua of spontaneous abortion cases might induce the low expression of IDO in DCs and monocytes. Munn *et al.* (2002, 2004) reported that specific populations of DCs only express IDO with IFN-γ treatment. Specific populations of DCs and monocytes which could express the IDO enzyme with IFN-γ or CTLA-4-/Fc treatment might increase in the pregnancy decidua, and the population of these DCs and monocytes might decrease in spontaneous abortion.

In this study, we studied the expression of IDO as determined by flow cytometry. We should check whether these results are correlated with IDO activity or not. We studied the concentration of kynurenine, the major IDO degradation byproduct [using high-performance liquid chromatography (HPLC) analysis], in the conditioned media following *in vitro* culture of the peripheral blood mononuclear cells and decidual leukocytes. IDO-mediated tryptophan degradation in both peripheral and decidual leukocytes were up-regulated during pregnancy. On the other hand, both kynurenine concentration after IFN-γ treatment or CTLA-4 treatment were decreased in spontaneous abortion cases suggesting that IDO expression determined by flow cytometry is well correlated with the IDO enzyme activity.

In conclusion, our data showed that IDO expression in decidual monocytes and DCs by CTLA-4/Fc or IFN-γ treatment were increased in therapeutic abortion decidua but were decreased in spontaneous abortion. CTLA-4 on Treg cells might play a role in the maintenance of pregnancy by the induction of IDO in DCs and monocytes.

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Submitted on August 23, 2005; accepted on November 11, 2005

PTEN and p53 abnormalities are indicative and predictive factors for endometrial carcinoma

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Received August 20, 2004; Accepted October 18, 2004

Abstract. PTEN (phosphatase and tensin homologue deleted on chromosome 10) and p53 alterations were expected to be diversely involved in endometrial carcinogenesis. Patients (n=92) with endometrial carcinoma (EC) were analyzed, and PTEN and p53 were immunostained in the tissue sections. Tumor histology, grade of differentiation, presence of endometrial hyperplasia, staining status of PTEN and p53 and clinical information were examined. There were 37 cases (40%) negative for PTEN staining, which suggests lost or reduced PTEN function. Loss of PTEN staining was significantly related to the advanced staging in the grade 1 (G1) and grade 2 (G2) endometrioid adenocarcinoma group (p=0.026). Also, 18 cases (20%) showed positive staining for p53. p53 staining was largely found in grade 3 (G3) endometrioid adenocarcinoma and other phenotypes of EC. In the G1 and G2 group, all 29 cases with reduced PTEN staining showed p53-negative staining (p=0.025). In the G3 and others group, 6 of 8 cases with reduced PTEN staining showed p53-positive staining. p53-positive staining was associated with a high probability of tumor recurrence in the G1 and G2 group (p=0.0234). In contrast, in the G3 and others group, p53-positive cases had a low probability of tumor recurrence (p=0.0473). Both PTEN and p53 staining may be good indicators of clinical stage and probability of tumor recurrence in EC. Reciprocal abnormality of p53 or PTEN occurred at an early phase of carcinogenesis, however simultaneous abnormality of p53 and PTEN often occurred at the a late phase of carcinogenesis. Thus, immunohistochemistry for PTEN and p53 in biopsy specimens of EC can provide supportive information for determining a treatment plan.

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Key words: immunohistochemistry, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), p53, endometrial carcinoma

Introduction

Uterine endometrial carcinoma is the fourth most frequent malignancy in females (1). Several genetic abnormalities were reported in endometrial carcinoma (2). Mutation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was one of the molecular abnormalities in endometrial carcinoma. A K-*ras* mutation was reported to be approximately 10-30% in endometrial carcinoma (3). A frequency of mutations in the ß-catenin gene was shown to be 14-44% in endometrial carcinoma (4). p53 abnormality, the most critical event leading to cancer in general, was also observed in endometrial carcinoma at 10-25% (5-7). It was reported that insulin-like growth factors (IGFs) played a role in mediating estrogen-induced endometrial proliferation, and therefore IGF signaling was a risk factor for endometrial carcinoma (8,9).

The hyperplasia-carcinoma sequence has been suggested in endometrial carcinogenesis. In accordance with the general classification of endometrial cancers, tumors with endometrial hyperplasia were categorized as type I, which mostly contains grade 1 (G1) and grade 2 (G2) endometrioid adenocarcinoma. Tumors without endometrial hyperplasia were categorized as type II, which contains mostly grade 3 (G3) endometrioid adenocarcinoma and other histological types, such as adenosquamous carcinoma, and serous, clear cell and mucinous adenocarcinoma (10). Type I tumors are known to be caused by excess hormonal stimulants, such as estrogen and/or progesterone relatives (11,12). Type II tumors are generally recognized as developing from atrophic endometrial tissue in older women and are independent of hormonal stimulation (11,13,14). Risk factor(s) for type II tumors remain unknown. Type I tumors are associated with mutations in the K-*ras* as well as the PTEN gene (11). They often have microsatellite instability, but do not usually possess mutations in the p53 gene (11). In contrast, type II tumors mostly have p53 mutations, but seldom have microsatellite instability or K-*ras* or PTEN mutations (11).

PTEN was first identified as a tumor suppressor gene located in 10q23, and the mutations were widely distributed in cancers ranging from brain to prostate (15). It was soon revealed that PTEN was responsible for Cowden's disease, a cancer predisposition syndrome (16). Although PTEN mutations were found predominantly in advanced cancers in

Table I. Clinicopathological characteristics of patients with endometrial carcinoma.

Characteristics	No. of patients	Type I[a]	Type II[a]	p-value
Total analyzed	92	49	43	
Histology group[b]				0.043
G1 and G2	67	40	27	
G3 and others	25	9	16	
FIGO[c] stage				0.019
I	56	37	19	
II	12	5	7	
III	20	6	14	
IV	4	1	3	
Age (years)				0.046
<60	61	37	24	
≥60	31	12	19	
Menopause				<0.001
(-)	30	24	6	
(+)	62	25	37	
Pregnancy				0.964
(-)	13	7	6	
(+)	79	42	37	
Prognosis				0.566
Dead	7	3	4	
Alive	85	46	39	

[a]Type I, endometrial carcinoma with endometrial hyperplasia; type II: endometrial carcinoma without endometrial hyperplasia. [b]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma. [c]International Federation of Gynecology and Obstetrics.

Table II. Relationship between histological characteristics and clinical stage.

Histological characteristics	FIGO[a] stage I	II	III	IV	p-value
Differentiation[b]					0.085
G1	24	4	4	0	
G2	23	5	6	1	
G3	5	1	6	2	
Adenosquamous	3	2	4	0	
Serous	1	0	0	1	
Histology group[c]					0.004
G1 and G2	47	9	10	1	
G3 and others	9	3	10	3	

[a]International Federation of Gynecology and Obstetrics. [b]G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3; Adenosquamous, adenosquamous carcinoma; Serous, serous adenocarcinoma. [c]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others: endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

general, it was reported that mutations occurred as an early event in endometrial carcinogenesis (2,17-19). Endometrial hyperplasia as well as endometrial carcinoma have been shown to have PTEN mutations in 20-30% and 30-80% of cases, respectively (2,17-23). The significance of PTEN mutations in endometrial carcinoma is interpreted in two opposite and conflicting ways. One interpretation is that PTEN alterations are related to a better prognosis (24,25), and the other is that the mutations result in a poor survival rate (22,23,26). Thus, clinical significance of the PTEN abnormality in endometrial carcinoma is not fully understood.

The tumor suppressor protein p53 plays an important role in mediating a response to stress, such as that induced by DNA damage or hyperproliferative signals resulting in either growth arrest or apoptosis (27,28). It was reported that a p53 abnormality relates to a later stage in endometrial carcinogenesis (24,25,29,30). Singh *et al* reported that simultaneous abnormality in PTEN and p53 were rare in head and neck squamous cell carcinoma (31). They also suggested that activation of phosphatidylinositol-3-kinase (PI3K) and mutation of p53 were mutually exclusive events, and either event is able to promote a malignant phenotype of the tumor (31). However, in the endometrial carcinoma, the examination of abnormalities in both PTEN and p53 pathways at the same time, using clinical materials, has not previously been performed.

In this study, we investigated abnormalities of PTEN and p53 in human endometrial carcinoma by immunohistochemistry, and examined the relationship of the abnormality of PTEN with that of p53 in endometrial carcinoma. Moreover, we analyzed the clinical significance of PTEN and p53 abnormalities in endometrial carcinoma.

Materials and methods

Cases and tissue samples. Tissue specimens of 92 patients who underwent surgery for endometrial carcinoma at Dokkyo University School of Medicine were analyzed. The clinical stage of the cancer progression was estimated according to the International Federation of Gynecology and Obstetrics (FIGO) 1988 criteria (32). Surgically-resected tissues were used for hematoxylin and eosin staining. Histological diagnosis, differentiated grade, depth of cancer invasion, and presence or absence of hyperplasia of the adjacent endometrium were evaluated based on the Armed Forces Institutes of Pathology (AFIP) classification (10).

Cell culture. Ishikawa cells (3-H-12-No107) were kindly provided by Dr M. Nishida (Kasumigaura National Hospital, Tsuchiura, Ibaraki, Japan) and HEC-1-A cells were purchased from American Type Culture Collection (Manassas, VA, USA). These cell lines were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Sigma) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA), 200 mmol/l

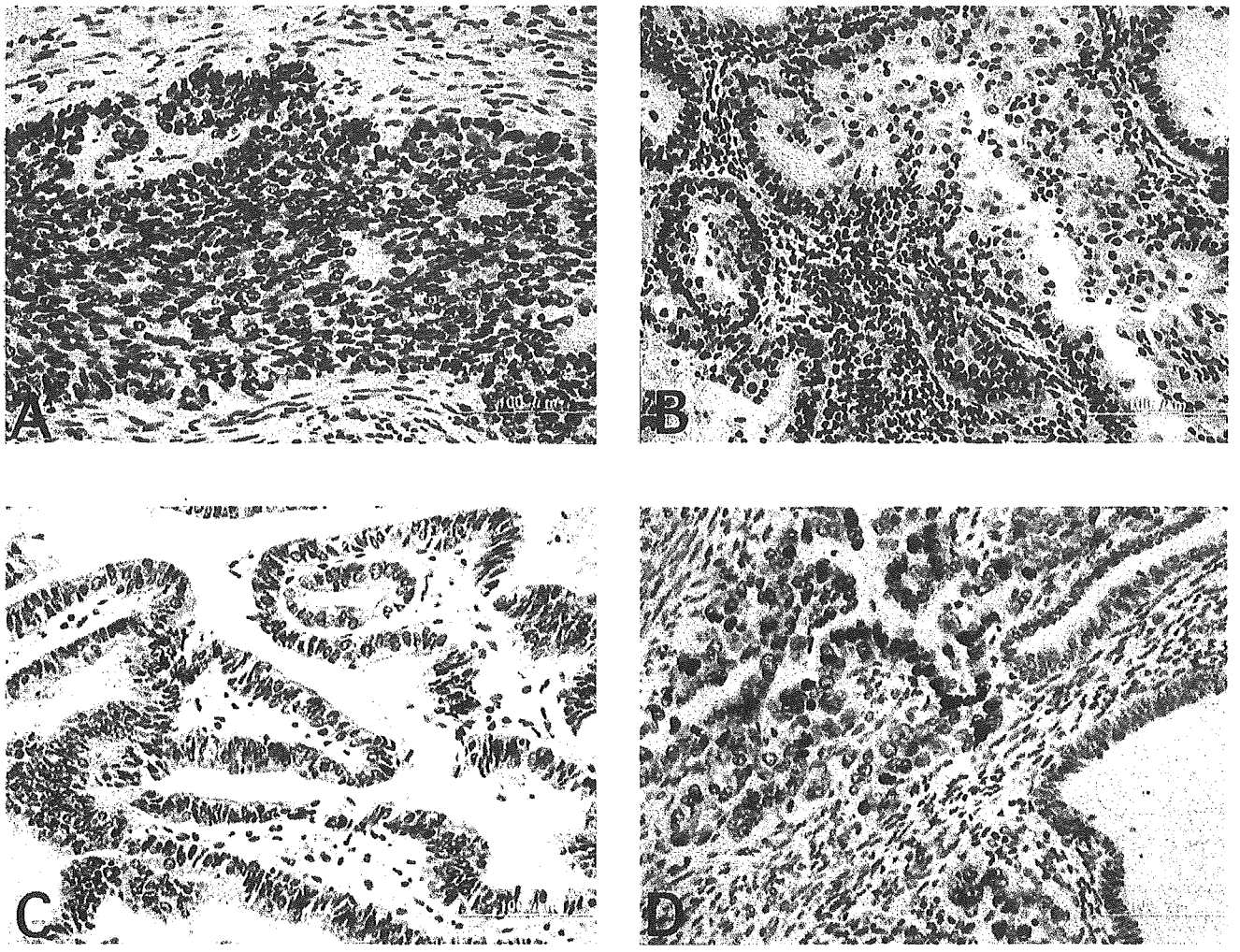

Figure 1. PTEN or p53 expression in endometrial carcinoma. (A) Abundant expression of PTEN. (B) Loss of PTEN expression. (C) Reduced PTEN expression. These three cases represent grade 3 (G3) endometrioid adenocarcinoma (A), G2 endometrioid adenocarcinoma (B), and G1 endometrioid adenocarcinoma (C). (D) p53-positive staining in G2 endometrioid adenocarcinoma; p53 positive-staining in the nuclei of the cancer cells are observed, in contrast to negative staining of the hyperplastic glands (lower right).

L-glutamine, and penicillin/streptomycin at 37°C in 95% air-5% CO_2. For immunohistochemical staining, cell pellets were fixed by 10% neutralized formaldehyde and embedded in paraffin as proceeded for tissue samples.

Immunohistochemical staining. Sections (4 μm-thick) were mounted on poly-L-lysine coated slides and deparaffinized in xylene and rehydrated through a series of graded alcohol. Antigen retrieval was performed for 10 min at 95°C in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Sections were also incubated with Protein Block Serum-Free solution (Dako, Carpenteria, CA) in order to block non-specific staining, according to the manufacturer's protocol. Anti-PTEN antibody (28H6; Novocastra, Balliol Business Park West, UK) and anti-p53 antibody (CM1; Novocastra) were used at 1:100 dilution for 60 min at room temperature, respectively. As a negative control, pre-immune serum was used instead of the specific antibodies to verify the specificity. The sections were washed with phosphate-buffered saline (PBS) 3 times each for 5 min. The Dako LSAB2 kit was used, based on the manufacturer's protocol (Dako), followed by PBS washing 3 times. Visualization was performed by immersing 3-3'-diaminobenzidine in chromogen substrate for 1 min. The stained slides were counterstained with hematoxylin and cover-slipped with EUKITT (O. Kindler, Freiburg, Germany).

Evaluation of the immunohistochemical staining. The status of PTEN staining was evaluated based on the staining intensity and distribution. Intensity was judged as strong, moderate, or weak. Distribution was scored as diffuse (<50% tumor staining), regional (15-50% tumor staining), and focal (<15% tumor staining). Tumors with intense to diffuse, intense to regional, intense to focal, and moderate to diffuse staining were considered positive for PTEN expression. Whereas tumors with moderate to regional, moderate to focal, or weak staining with any distribution were considered negative for PTEN expression.

For p53 staining, cases were defined as positive when >10% tumor cells showed strong nuclear staining.

Statistical analysis. Using Pearson's Chi-square test, the abnormalities of PTEN and/or p53 were assessed for their

Table III. Implication of the abnormality of PTEN with clinicopathological characteristics of the patients.

Clinicopathological characteristics	No. of patients	Expression of PTEN		p-value
		Reduced	Normal	
Total analyzed	92	37	55	
Tumor type[a]				0.114
Type I	49	16	33	
Type II	43	21	22	
Age (years)				0.255
<60	61	22	39	
≥60	31	15	16	
FIGO[b] stage				0.206
I	56	19	37	
II	12	8	4	
III	20	8	12	
IV	4	2	2	
Differentiation[c]				0.151
G1	32	13	19	
G2	35	16	19	
G3	14	2	12	
Adenosquamous	9	4	5	
Serous	2	2	0	
Histology group[d]				0.326
G1 and G2	67	29	38	
G3 and others	25	8	17	

[a]Type I, endometrial carcinoma with endometrial hyperplasia; type II: endometrial carcinoma without endometrial hyperplasia. [b]International Federation of Gynecology and Obstetrics. [c]G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3; Adenosquamous, adenosquamous carcinoma; Serous, serous adenocarcinoma. [d]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

association with different clinical and pathologic parameters including clinical stage, tumor histological grade, and recurrence-free probability (RFP). The RFP was estimated using the Kaplan-Meier method, and compared using the log-rank test. All statistical analyses were performed using SPSS II software (version 11.0.1 for Windows; SPSS, Inc., Chicago, IL). $p<0.05$ was considered statistically significant.

Results

Clinicopathological characteristics of the patients. A total of 92 patients with endometrial carcinoma (age range, 31-82; mean age, 57) were examined. In our study, there were 49 cases of type I tumors, and 43 cases of type II tumors. Type I was largely composed of G1 and G2 endometrioid adenocarcinoma, and type II composed of G3 endometrioid adenocarcinoma and others (p=0.043, Table I). Type II tumors were found mostly in advanced clinical stages, and type I tumors were less advanced (p=0.019, Table I). Type I tumors occurred predominantly in young women with menstrual cycles (p=0.046 and p<0.001, respectively, Table I). Degree of cancer differentiation had a tendency towards a progressed clinical stage, although its relationship did not reach a significant level (p=0.085, Table II). However, when combining the G1 and G2 groups, and the G3 and others groups, the latter histological group was frequently found in an advanced clinical stage of the disease (p=0.004, Table II).

Table IV. Implication of the abnormality of PTEN with clinicopathological characteristics in the G1 and G2 histology group.

Clinicopathological characteristics	No. of patients	Expression of PTEN		p-value
		Reduced	Normal	
FIGO[a] stage				0.026
I	47	15	32	
II	9	7	2	
III	10	6	4	
IV	1	1	0	
Total	67	29	38	

[a]International Federation of Gynecology and Obstetrics.

Implication of the abnormality of PTEN with clinicopathological characteristics of the patients. We first tested the specificity of the 28H6 antibody for staining PTEN protein in formalin-fixed, paraffin-embedded samples. Ishikawa cells are reported to have two-point mutations in the PTEN gene, and both mutations produced the stop codon (33). On the other hand, HEC-1-A cells are reported to have the wild-type PTEN gene (33). The 28H6 antibody showed a negative result for Ishikawa cells and a positive result for HEC-1-A cells (data not shown). Therefore, we used the 28H6 antibody for further immunohistochemical study.

As shown in Fig. 1A, there were cancer cells possessing abundant PTEN expression in the nuclei. In contrast, there were cells in which staining for PTEN was dramatically reduced (Fig. 1B) or moderately decreased (Fig. 1C). After evaluating the staining status according to its area and intensity (see Materials and methods), 37 cases (40%) were judged as negative for PTEN, which suggests lost or reduced PTEN function in the cells. No significant relationship was observed between PTEN abnormalities and endometrial hyperplasia, age, clinical stage, or histology and degree of cancer differentiation (Table III). Moreover, the expression of PTEN, in other words PTEN function, was not related to the histological group, G1 and G2, and G3 and others (Table III). However, PTEN expression was significantly reduced in the G1 and G2 group at an advanced stage (p=0.026, Table IV).

Table V. Implication of the abnormality of p53 with clinicopathological characteristics of the patients.

Clinicopathological characteristics	No. of patients	Nuclear accumulation p53		p-value
		Positive	Negative	
Total analyzed	92	18	74	
Tumor type[a]				0.403
Type I	49	8	41	
Type II	43	10	33	
Age (years)				0.103
<60	61	9	52	
≥60	31	9	22	
FIGO[b] stage				0.345
I	56	11	45	
II	12	1	11	
III	20	4	16	
IV	4	2	2	
Differentiation[c]				<0.001
G1	32	2	30	
G2	35	4	31	
G3	14	5	9	
Adenosquamous	9	5	4	
Serous	2	2	0	
Histology group[d]				<0.001
G1 and G2	67	6	61	
G3 and others	25	12	13	

[a]Type I, endometrial carcinoma with endometrial hyperplasia; type II, endometrial carcinoma without endometrial hyperplasia. [b]International Federation of Gynecology and Obstetrics. [c]G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3; Adenosquamous, adenosquamous carcinoma; Serous, serous adenocarcinoma. [d]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

Table VI. Implication of the abnormality of p53 with clinicopathological characteristics in the G1 and G2 histology group.

Clinicopathological characteristics	No. of patients	Nuclear accumulation p53		p-value
		Positive	Negative	
FIGO[a] stage				0.423
I	47	6	41	
II	9	0	9	
III	10	0	10	
IV	1	0	1	
Total	67	6	61	

[a]International Federation of Gynecology and Obstetrics.

Table VII. Relationship between PTEN and p53 staining.

	Nuclear accumulation p53		p-value
	Positive	Negative	
Expression of PTEN in total cases (92 cases)			0.507
Reduced	6	31	
Normal	12	43	
Expression of PTEN in the G1 and G2 group[a] (67 cases)			0.025
Reduced	0	29	
Normal	6	32	
Expression of PTEN in the G3 and others group[a] (25 cases)			0.064
Reduced	6	2	
Normal	6	11	

[a]G1 and G2, endometrioid adenocarcinoma grades 1 and 2; G3 and others, endometrioid adenocarcinoma grade 3 and other histological types, adenosquamous carcinoma and serous adenocarcinoma.

Implication of the abnormality of p53 with clinicopathological characteristics of the patients. As shown in Fig. 1D, nuclear p53 staining was confirmed in the cancer cells. There were 18 cases (20%) that showed positive staining for p53, while the remaining 74 cases (80%) were negative. There was no significant relationship between p53-positive staining and the presence or absence of endometrial hyperplasia, age distribution, or clinical stage (Table V). On the other hand, p53-positive staining was largely found in the G3 and others group (p<0.001, Table V). In contrast, there was no relationship between p53-positive staining and clinical stage in the G1 and G2 group (p=0.423, Table VI).

Relationship of the PTEN abnormality with the p53 abnormality. There was no significant relationship between the PTEN and p53 staining patterns in 92 cases of endometrial carcinoma (p=0.507, Table VII). However, in G1 and G2 group, there was significant relationship between the PTEN and p53 staining patterns (p=0.025, Table VII). All 29 cases with reduced PTEN staining pattern showed p53-negative staining (Table VII). All 6 cases with p53-positive staining pattern showed reduced PTEN staining (Table VII). In the G3 and others group, 6 of 8 cases with reduced PTEN staining pattern showed p53-positive staining, although it did not reach a significant level (p=0.064, Table VII).

Recurrence-free probability (RFP). In the G1 and G2 group, PTEN abnormality was not associated with tumor recurrence

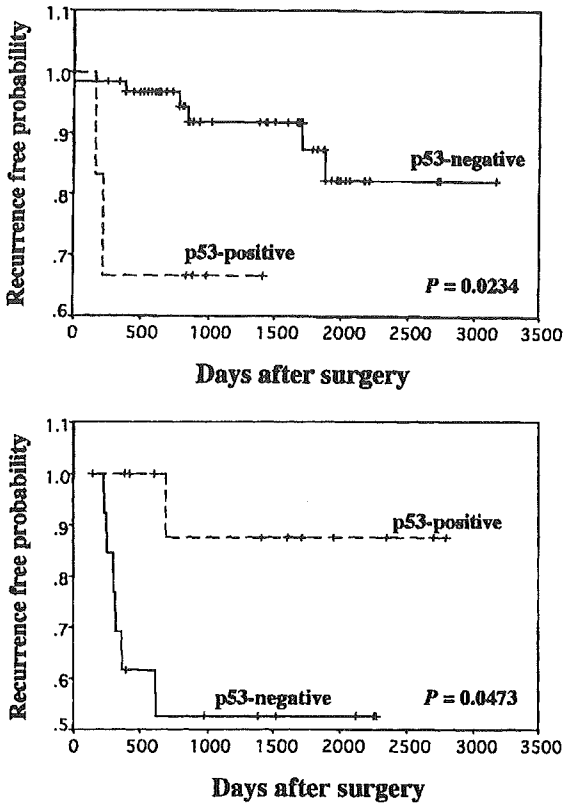

Figure 2. (A) Recurrence-free probability (RFP) for patients who had G1 and G2 endometrioid adenocarcinoma (G1 and G2 group) with or without a p53 abnormality. (B) RFP for patients who had G3 endometrioid adenocarcinoma and other phenotypes (G3 and others group) with or without a p53 abnormality.

(p=0.3149, data not shown), whereas patients with p53-positive staining showed a lower RFP than those without p53 staining (p=0.0234, Fig. 2A). In contrast, in the G3 and others group, patients with p53-positive staining had a higher RFP than those without p53 staining (p=0.0473, Fig. 2B). When we compared the PTEN abnormality or p53 abnormality and RFP in all histological types, no significant relationship was observed (data not shown).

Discussion

In this study, we demonstrated that loss of PTEN staining was significantly related to the advancement of disease staging in the G1 and G2 group, in which most tumors were categorized as type I tumors. Moreover, p53-positive staining was largely observed in the G3 and others group, in which most tumors were categorized as type II tumors. Furthermore, in endometrial carcinogenesis, reciprocal abnormality of p53 or PTEN occurred at an early phase of carcinogenesis, however simultaneous abnormality of p53 and PTEN often occurred at a late phase of carcinogenesis. p53-positive staining was associated with a high probability of tumor recurrence in the G1 and G2 group. In contrast, in the G3 and others group, cases with p53-positive staining had a lower probability of recurrence than those without p53 abnormalities.

In this experimental condition, PTEN was stained in the nucleus. PTEN does not have a nuclear localization signal (15), however several studies concerning PTEN immunohistochemistry showed nuclear localization of the PTEN protein (34-36). Although it was reported that phosphorylation of the PTEN protein would decrease PTEN activity and the phosphorylated-PTEN localized in the nucleus, most of the investigators evaluated the nuclear staining of PTEN as evidence of the normal function of PTEN protein. Gimm *et al* (37) reported that their monoclonal antibody 6H2.1 specifically recognized the 55 kDa protein only in cells with a normal PTEN gene, and the monoclonal antibody detected nuclear localization of the PTEN protein in several cells. They also confirmed that an absorption test using PTEN peptides completely abolished immunostaining with this antibody (37). Although an antibody we used was different from that of Gimm *et al*, clear nuclear staining of PTEN appeared to reflect the normal function of the PTEN protein.

The status of PTEN staining was evaluated based on the staining intensity and distribution. Tumors with intense to diffuse, intense to regional, intense to focal, and moderate to diffuse staining were considered positive for PTEN expression (normal PTEN function). Whereas tumors with moderate to regional, moderate to focal, or weak staining with any distribution were considered negative (dysfunction of PTEN: genetic deletion, truncation protein producing mutation, and down-regulation of gene expression). Some of the PTEN-negative cases were confirmed to have a genetic deletion or truncation protein-producing mutation in the PTEN gene (unpublished data).

For p53 staining, cases were defined as positive when >10% tumor cells showed strong nuclear staining. In our previous experiments (38-41), p53-positive staining tumor cells in other organs, such as colon, esophagus, gallbladder, and head and neck were confirmed to have a p53 missense mutation.

It was reported that AKT enhances MDM2-mediated ubiquitilation and degradation of wild-type p53 (42) and, more recently, that PTEN and PI3K inhibitor up-regulate p53 and block tumor-induced angiogenesis in glioma cells (43). Thus, PTEN activity up-regulates the wild-type p53 function via an inhibition of AKT-mediated MDM2 activation. In contrast, Stambolic *et al* (44) have reported that wild-type p53 directly binds to the promoter sequence, and enhances expression of the PTEN gene. Thus, the p53 and PTEN pathways have a cross-talk in their signaling pathway. In head and neck squamous cell carcinoma, Singh *et al* (31) reported that activation of PI3K (down-regulation of PTEN function) and mutation of p53 were mutually-exclusive events.

In our study, there was a significant relationship between PTEN and p53 staining patterns in the G1 and G2 group. Interestingly, all 29 cases with reduced PTEN staining pattern in the G1 and G2 group showed p53-negative staining. Furthermore, all 6 cases with p53-positive staining pattern in the G1 and G2 group showed normal PTEN staining. However, in contrast to the G1 and G2 group, 6 of 8 cases with reduced PTEN staining pattern in the G3 and others group showed p53-positive staining. In endometrial carcinogenesis, reciprocal abnormality of p53 or PTEN occurred at an early phase of carcinogenesis, however simultaneous abnormality of p53 and PTEN often occurred at a late phase of carcinogenesis.

In the G1 and G2 group, 32 of 67 cases (48%) did not show either PTEN or p53 abnormalities in our experiment. These tumors may have other genetic abnormalities, such as

K-*ras* mutation (3), ß-catenin mutation (4), IGF overexpression (8,9), and MSH3 and MSH6 mutations (45). Moreover, it might be that several factors regulate PTEN expression in endometrial carcinoma. It was reported that ribonucleotide reductase M1 is able to up-regulate PTEN expression (46), and progesterone and estrogen were recently shown to be involved in PTEN regulation (47).

From a clinical aspect, it was very important that reduced PTEN staining was significantly related to the advancement of disease staging in the G1 and G2 group. In addition, a p53 abnormality was significantly related to poor prognosis in the G1 and G2 group, despite the fact that frequency was very low (9%). In contrast, in the G3 and others group, cases without p53-positive staining have better prognoses than cases with p53-positive staining. We previously demonstrated that in advanced cancer, tumors with p53 abnormality frequently showed p53-negative staining (48). These tumors always have a homozygous deletion of p53 gene or truncation protein-producing mutation. These tumors sometimes showed aggressive behavior when compared to tumors with missense mutated-p53, which showed p53-positive staining in immunohistochemistry. Thus, in the G3 and others group, p53-negative cases may have included such aggressive tumors, and showed poor prognosis.

In conclusion, both PTEN and p53 staining may be good indicators of clinical stage and probability of tumor recurrence in endometrial carcinoma. For women wishing to preserve their uterus, especially those who have not yet had a baby, it is a very serious decision when gynecological oncologists recommend hysterectomy. As an alternative treatment, curettage of endometrial tissue with or without hormonal therapy may be selected for patients at an earlier clinical stage. We would like to propose that immunohistochemistry for PTEN and p53 in biopsy specimens of endometrial carcinoma can provide supportive information for determining a treatment plan.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. We would like to thank Ms. Chiaki Matsuyama-Sato, Ms. Ayako Shimizu, Ms. Takako Ohtsuki, and Ms. Midori Matsuura for their excellent technical assistance.

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Gynecologic Oncology 97 (2005) 405–409

Gynecologic Oncology

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Inhibitory effects of herbal drugs on the growth of human ovarian cancer cell lines through the induction of apoptosis

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Received 25 August 2004
Available online 24 March 2005

Abstract

Objective. In order to develop and search for more effective and safe treatments for early and advanced stages of ovarian cancer, we examined the direct effects of four extracts of Chinese herbal drugs on ovarian cancer cells in vitro.

Methods. The growth inhibition of four herbal drugs on a total of six cell lines of human ovarian cancer cells was determined by a Cell Counting Kit-8 by counting viable cells. Apoptotic cells induced by herbal drugs were detected by using MEBCYTO Apoptosis Kit. All experiments were performed in triplicate. The significance of the difference was analyzed with a two-sided Student's t test. A P value less than 0.05 was accepted as statistically significant.

Results. The MN, A2780, and KF cell lines exhibited significant growth inhibition in the presence of Sho-saiko-to concentrations of 150 μg/ml, 300 μg/ml, and 500 μg/ml, respectively, and at the concentration of 1000 μg/ml, Sho-saiko-to demonstrated a significant apoptotic induction effect on all six kinds of ovarian cancer cell lines. This concentration is the same as the blood concentration attained when 7.5 g of Sho-saiko-to per day is orally administered and all absorbed.

Conclusions. Sho-saiko-to exhibited significant growth inhibition of ovarian cancer cell lines, and the mechanisms of the inhibitory effects can be attributed, in part, to apoptosis induced by Sho-saiko-to.

Keywords: Herbal medicine; Sho-saiko-to; Human ovarian cancer cell lines; Inhibitory effects; Apoptosis; Necrosis

Introduction

Ovarian cancer causes more deaths per year than any other cancer of the female reproductive system. In Japan, there has been a steady increase in newly diagnosed patients with ovarian cancer and patients dying from this disease in recent years. The currently used chemotherapy is relatively effective against ovarian cancer, but because of side effects or resistance to chemotherapeutic agents, the 5-year survival rate has not been dramatically improved. Consequently, the development and search for more effective and safe treatment modalities and/or adjuvant therapies for early and advanced stages of the disease have now become important research targets.

The use of Chinese herbal drugs, which have been used in China for thousands of years, has now been increasing in recent years [1] because of their safety and few side effects. Some herbal drugs have been reported to possess chemotherapeutic effects. For example, *Scutellaria baicalensis* exhibits effective anticancer activity on head and neck squamous cell carcinoma in vitro and in vivo [2], and *Euphorbia fischeriana* has inhibitory effects on the human prostate LNCaP cancer cell line [3].

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doi:10.1016/j.ygyno.2004.12.063

However, there are few reports published on potential effects of herbal drugs on ovarian cancer. In this study, we examined the direct effects of four Chinese herbal drugs on ovarian cancer cells in vitro. The aim of this study was to evaluate the effects of herbal drugs on the proliferation of cultured ovarian cancer cells in vitro and investigate their mechanism of action.

Materials and methods

Chemicals and drugs

The powder forms of four kinds of herbal medicine (Sho-saiko-to, Hochu-ekki-to, Juzen-taiho-to, and Ninjin-yoei-to) were obtained from Tsumura Co. (Tokyo, Japan) and were dissolved in RPMI 1640 medium up to 5000 μg/ml, vortexed at room temperature for 1 min, and incubated under rotation at 37°C for 1 h. These solutions were centrifuged at 5000 rpm for 5 min to remove any insoluble ingredients. The supernatants were passed through a 0.22-μm filter for sterilization.

Cell lines and tissue culture

A total of six cell lines of human ovarian cancer were used in the present study. KF-1, MN-1, A2780 and their respective cisplatin-resistant sublines KF-r, MN-r, A2780cp cell lines were provided by Dr. Y. Kiyozuka (Department of Pathology II, Kansai Medical University, Osaka, Japan). These cell lines were cultured in RPMI 1640 medium (Sigma Chemical corporation, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical corporation, USA) and 1% penicillin–streptomycin (Invitrogen Corporation, USA). All cell lines were cultured at 37°C in 5% CO_2/95% air.

Cell viability assay

The growth inhibition of the four herbal drugs on ovarian cancer cells was determined by counting viable cells with a Cell Counting Kit-8 (Dojindo, Japan). An equal number of cells of the six cell lines (1×10^4 cells/well) in 100 μl of conditioned medium were seeded into a 96-well microplate and incubated overnight at 37°C in 5%CO_2/95% air. Then, the cells were treated with various concentrations of Sho-saiko-to, Ninjin-yoei-to, Hochu-ekki-to, and Juzen-taiho-to diluted in 100 μl of conditioned medium (the final concentrations of the four herbal drugs were 25, 50, 100, 200, 500, 1000, and 5000 μg/ml). After incubation for 48 h, 10 μl of Cell Counting Kit-8 solution were added to each well, and the plates were further incubated for 4 h at 37°C. The absorbance at 460 nm was measured with an ImmunoReader NJ-2000. Dose–response curves were plotted on a semi-log scale as the percentage of the control cell number, which was obtained from the sample with no drug exposure.

Cell apoptosis assay

Cell apoptosis induced by herbal drugs was detected by using MEBCYTO Apoptosis Kit (MBL CO., LTD. Nagoya, Japan). A total of 2×10^5 cells/well of the six cell lines were seeded into 12-well microplates for 24 h incubation at 37°C. Various concentrations of the four drugs, diluted in 100 μl of conditioned medium, were added to the wells and incubated for an additional 48 h (the final concentrations of each of the herbal drugs were 200, 1000, and 5000 μg/ml). After trypsinizing and gently washing cells once with medium, the cells were washed with PBS, and then resuspended in 85 μl of binding buffer. Ten microliters of Annexin V-FITC and 5 μl of propidium iodide were added to the resuspended cells. After incubation at room temperature for 15 min in the dark, 400 μl of binding buffer was added to the resuspended cells, and then the stained cells were analyzed by flow cytometry using a single laser emitting excitation light at 488 nm.

Statistical analysis

All experiments were performed in triplicate and the results of a representative experiment are presented. The significance of the difference was analyzed with a two-sided Student's t test. A P value less than 0.05 was accepted as statistically significant.

Results

Growth inhibition of herbal drugs on ovarian cancer cell lines

We first examined the direct effects of four kinds of herbal medicine on the growth of six ovarian cancer cell lines in vitro. The viability of the treated cell lines was determined as the ratio between viable treated cells and viable untreated control cells. As shown in Fig. 1A, Sho-saiko-to displayed direct anti-tumor effects on all six cell lines but at different concentrations. The MN, A2740, and KF cell lines exhibited significant growth inhibition at Sho-saiko-to concentrations of about 150 μg/ml, 300 μg/ml and 500 μg/ml, respectively. There was no significant difference in the degree of growth inhibition between parent and resistant CDDP sublines. The IC_{50} of the three cell lines were 189 μg/ml (95%CI 149–229), 513 μg/ml (95%CI 303–723), and 733 μg/ml (95%CI 503–963), respectively. On the other hand, Ninjin-yoei-to, Hochu-ekki-to, and Juzen-taiho-to did not inhibit the growth of any of the six cell lines even at concentrations of 1000 μg/ml (Figs. 1B–D), but when the concentration reached 5000 μg/ml, all three kinds of herbal medicine showed inhibitory effect on all six cell lines.

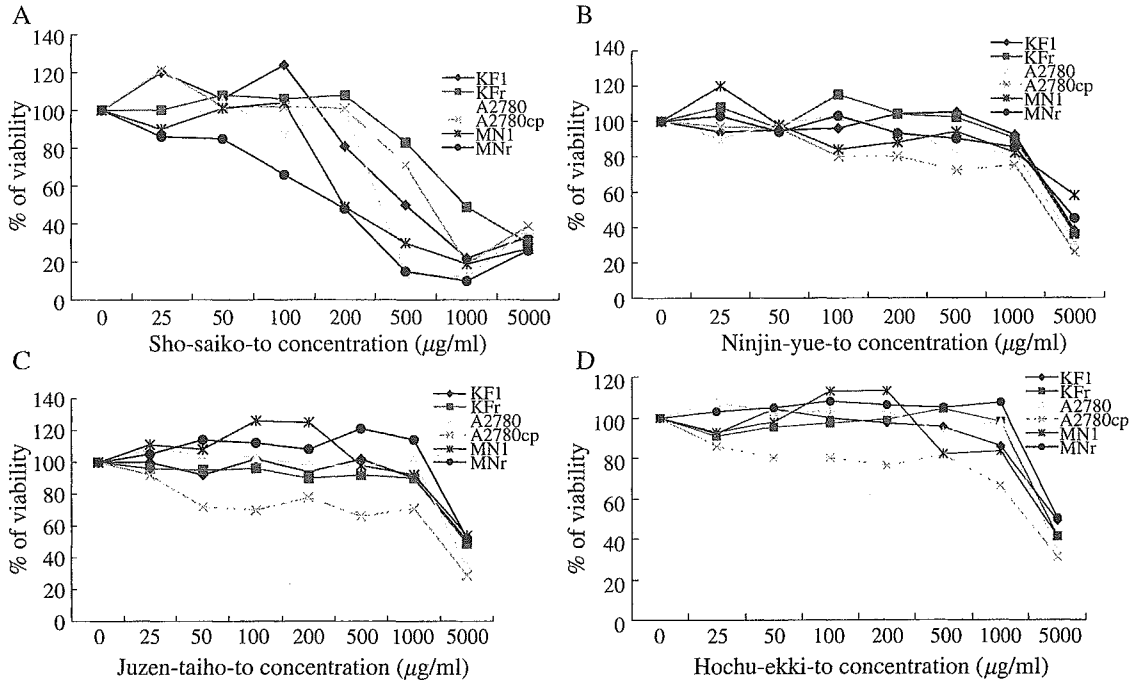


Fig. 1. Inhibitory effects of four kinds of medicine on the growth of human ovarian cancer cell lines. (A) Sho-saiko-to. (B) Ninjin-yoei-to. (C) Juzen-taiho-to. (D) Hochu-ekki-to. Cells were treated with various concentrations (0, 25, 50, 100, 200, 500, 1000, and 5000 μg/ml) of four herbal drugs for 48 h. The cell viability was determined by the Cell Counting kit as described in the text. Each data point represents the mean ± SD of results from four individual measurements.

Cell apoptosis assay

The MEBCYTO Apoptosis Kit is intended for the detection of early stages of apoptosis. The difference between apoptosis and necrosis is that, during the initial stages of apoptosis, the cell membrane remains intact, while at the very moment that necrosis occurs, the cell membrane loses its integrity and becomes leaky. Therefore, necrotic cells are easily stained with propidium iodide (PI) as well as Annexin V, whereas apoptotic cells are stained only with Annexin V. As shown in Fig. 2, cells in the lower right area (LR) indicate apoptotic cells, and the cells in the upper right area (UR) are necrotic cells induced by herbal medicine. In order to determine the mechanism of inhibition of herbal medicine on the ovarian cancer cell lines, the apoptotic effects of concentrations of 0, 200, 1000, and 5000 μg/ml were examined. From Fig. 3 and Fig. 4 (Fig. 4, only Juzen-taiho-to was given), it is apparent that, at 1000 μg/ml, Sho-saiko-to demonstrated a significant apoptotic effect on all six cell lines, whereas the other three kinds of herbal medicine did not exhibit any apoptotic effect. At a concentration of 5000 μg/ml, all four kinds of herbal medicine produced necrosis in all six cell lines. These results suggest that Sho-saiko-to suppresses the growth of the ovarian cancer cell lines through the induction of apoptosis.

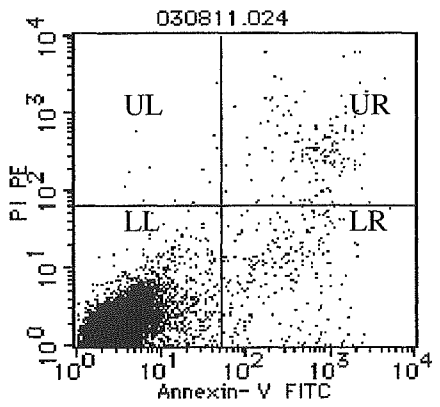
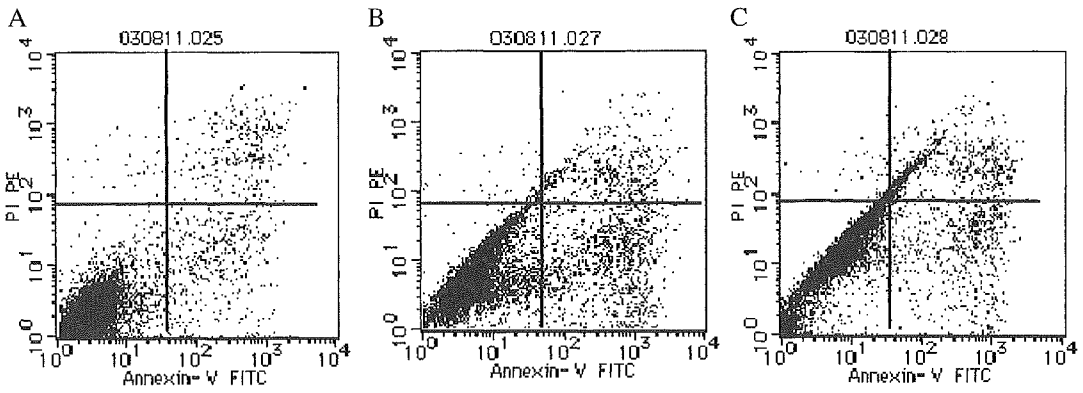


Fig. 2. Flow cytometry analysis of the cells treated with herbal drugs. LL: live cells; LR: apoptotic cells; UR: necrotic cells; UL: necrotic cells and debris; PI: propidium iodide.

Discussion

The Chinese herbal medicine Sho-saiko-to is a drug extract preparation comprising of seven herbal components (Bupleurum root, Pinellia tuber, Scutellaria root, Jujube fruit, Gingseng root, Glycyrrhiza root, and Ginger rhizome), and it is one of the most commonly used types of "Kampo" medicine that is being prescribed mainly for chronic liver disease in Japan. Sho-saiko-to is indicated for improvement of liver dysfunction due to chronic hepatitis [4–7]. Recently, cancer-preventive [8] and anti-tumor effects of Sho-saiko-to on human hepatoma cell lines [9–13] and tumor trans-



Control (0 μg/ml)

Quad	%Gated
UL	0.38
UR	2.99
LL	93.00
LR	3.63

1000l μg/ml

Quad	%Gated
UL	0.96
UR	4.35
LL	84.62
LR	10.07

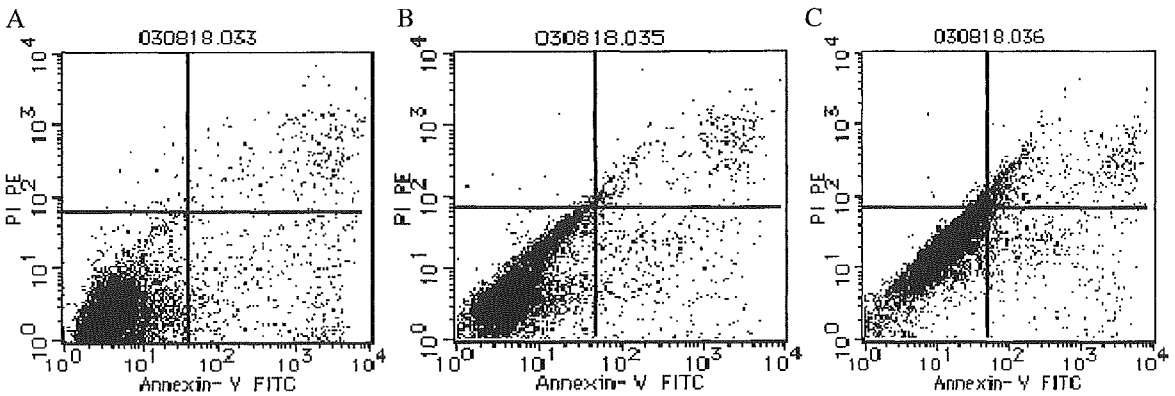
5000 μg/ml

Quad	%Gated
UL	4.06
UR	6.95
LL	83.44
LR	5.55

Fig. 3. Flow cytometry analysis of MN cell line treated with Sho-saiko-to. (A) Untreated control. (B) At the concentration of 1000 μg/ml, Sho-saiko-to demonstrated a significant effect on the induction of apoptosis in the MN cell line compared with control cells. (C) At the concentration of 5000 μg/ml, Sho-saiko-to displayed a necrotic effect on the MN cell line compared with control cells. The necrotic effects of Sho-saiko-to on the other five cell lines were similar.

planted animals [14,15] have been reported. However, it remained unknown whether Sho-saiko-to affected ovarian cancer cell lines. In the present study, our results showed that Sho-saiko-to displayed anti-tumor effects on all six kinds of ovarian cancer cell lines at a concentration of 1000 μg/ml. Among the components of Sho-saiko-to, Glycyrrhizin, Baicalin, and Baicalein were reported to suppress the proliferation of carcinoma cell lines in vitro [10–13]. It is therefore reasonable to assume that the direct inhibitory effect of Sho-saiko-to on the ovarian cancer cell lines shown in our study may be caused by those active ingredients and perhaps additional as yet unknown ingredients. At the concentration of 1000 μg/ml, the inhibitory effect of Sho-saiko-to appears to involve apoptosis. This concentration (1000 μg/ml) is the same as the blood concentration attained when 7.5 g of Sho-saiko-to per day is orally administered and all absorbed. At the high concentration of 5000 μg/ml, Sho-saiko-to produced cell death via necrosis.

The mechanism of the apoptosis induced by Sho-saiko-to in the ovarian cancer cell lines remains unclear. In the



Control (0 μg/ml)

Quad	%Gated
UL	0.37
UR	2.35
LL	92.31
LR	4.97

1000 μg/ml

Quad	%Gated
UL	0.94
UR	2.98
LL	92.93
LR	3.15

5000 μg/ml

Quad	%Gated
UL	3.42
UR	6.78
LL	85.34
LR	4.46

Fig. 4. Flow cytometry analysis of MN cell line treated with Juzen-taiho-to. (A) Untreated control. (B) At the concentration of 1000 μg/ml, Juzen-taiho-to did not demonstrate a significant apoptotic effect on the MN cell line compared with control cells. (C) At the concentration of 5000 μg/ml, Juzen-taiho-to displayed a necrotic effect on the MN cell line compared with control cells. The necrotic effects of Juzen-taiho-to on the other five cell lines and those of Ninjin-yoei-to and Hochu-ekki-to on the six cell lines were similar.

hepatocellular carcinoma cell line, Sho-saiko-to acts as a biological response modifier and suppresses the proliferation of hepatoma cells in vitro by inducing apoptosis and arresting the cell cycle. It induces apoptosis in the early period of exposure and induces arrest at the G0/G1 phase in the late period of exposure [16]. The active ingredient, Baicalein inhibited the activity of topoisomerase II and induced apoptosis [17]. But in a murine malignant melanoma cell line (Mel-ret), Sho-saiko-to induced apoptotic cell death of Mel-ret cells with a definite increase of cell surface Fas antigen and Fas ligand [18]. Recently, Baicalin has been reported to act as a pro-oxidant and to induce caspase-3 activation and apoptosis via a mitochondrial pathway in a leukemia-derived T cell line [19]. These results indicate that several mechanisms may be involved in apoptosis induced by Sho-saiko-to in different kinds of malignant cell lines.

In conclusion, we have demonstrated, for the first time, that Sho-saiko-to exhibits a direct inhibitory effect on human ovarian cancer cell lines. The inhibitory effect can be attributed, in part, to apoptosis induced by Sho-saiko-to. These results may suggest potential therapeutic applications. Further investigations on its possible clinical applications in ovarian cancer therapy should be carried out.

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診療

サイトメガロウイルス（CMV）感染胎児に対する胎内治療と治療効果判定

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古谷健一* 菊池義公*

先天性サイトメガロウイルス（CMV）感染症症候性胎児の確定診断は，羊水もしくは胎児体液中のCMV DNAを同定することが必要とされるが，便宜的に母体血清IgMの上昇がスクリーニングに使用されている。しかし，母体血清IgM値はかならずしも胎内環境を反映するとは限らないことが臨床上問題となっている。今回，リアルタイムPCR法を用い，胎児体液中CMV DNAを同定，定量し，CMV抗体高力価γグロブリンによる胎内治療を施行，あわせて治療効果を判定し，良好な予後を得た症例を経験したので若干の文献的考察を加え報告する。

はじめに

Cytomegalovirus（CMV）による先天性CMV感染症は，TORCH症候群のひとつで，低出生体重児，肝脾腫，黄疸，脈絡網膜炎，脳内石灰化，側脳室石灰化，難聴などの重篤な症状を引き起こすことが知られている。新生児期に先天性サイトメガロウイルス感染症と診断された児では，症候性感染児では約80%で予後重篤であり[1]無症候性感染児でも約10～15%に重篤な神経学的障害をもたらす[2)3)]とされ，胎児期における確定診断と胎内治療が模索されている。

胎内における確定診断の正確さについては議論があったが，近年開発されたリアルタイムPCR法を用いたCMV DNA定量法は定性法に比べ格段に精度が高く，現在ではもっともすぐれた診断法と考えられる。母体CMV IgMによるスクリーニングはかならずしも胎内環境を反映するとは限らず，時間的にペア血清検査を施行できない場合やウイルスの再活性化によって引き起こされたと考えられる病態には対応できない可能性がある[4]。

一方，妊婦におけるCMV抗体保有率の調査では，近年わが国においても若年者ほど抗体保有率は低下しており[5)6)]，ワクチンが実用化されてない現状では，今後，欧米諸国なみに先天性CMV感染症の問題は注目されうると考えられる。

今回，胎児水腫にて紹介受診し，リアルタイムPCR法で胎児体液中CMV DNAを同定，定量し，CMV抗体高力価γグロブリンによる胎内治療を施行，あわせて治療効果を判定し，健常児を得た症例を経験した。胎内診断，胎内治療において有用と思われる知見を得たので若干の文献的考察を加え報告する。

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I. 症　例

26歳，0経妊0経産。家族歴，既往歴に特記事項なし。妊娠27週時に風邪症状を自覚。近

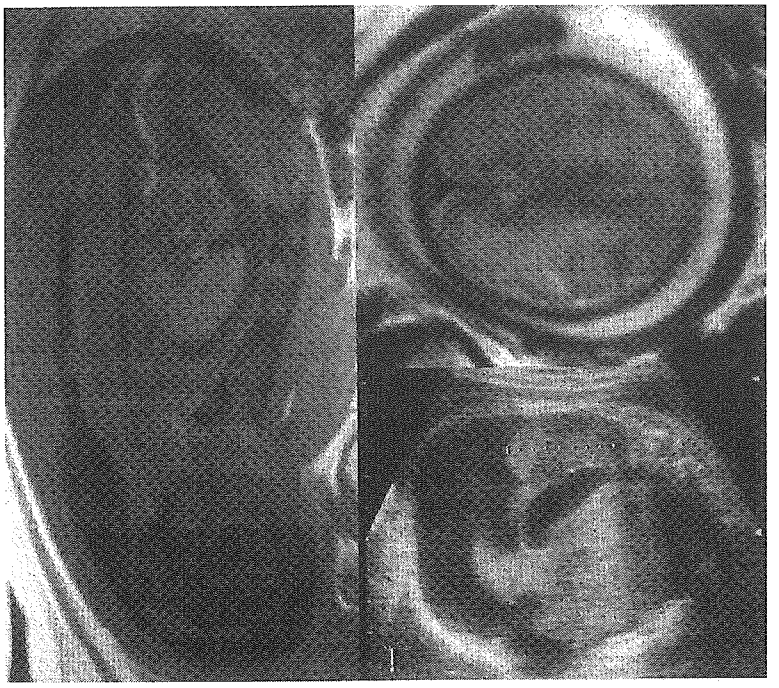

図 1　MRI 検査（左，右上）と超音波検査（右下）

医にて胎児腹水を指摘され，2001 年 7 月，妊娠 29 週 3 日に紹介入院となる。

1. 入院時検査

ノンストレステスト：Reassuring。

血液型：O 型 Rh(+)，不規則抗体検査陰性。

感染症検査：CMV IgG 16.0（EIA cut off：<2.0），CMV IgM 1.84（EIA cut off：<0.8），CMV DNA 1.8×10^2 copy/ml。パルボウイルス，風疹，梅毒，トキソプラズマは陰性。

超音波検査：単胎妊娠であり，胎盤，羊水量に異常なし。胎児腹水，肝脾腫を認める。脳内石灰化は認めず。その他の胎児奇形を認めず。推定体重 1520 g（腹水込）。ドップラー血流検査上 UmArPI/RI，MCAPI/RI，PLI など異常を認めず。

胎児 MRI：脳内に異常所見なし。胎児腹水，肝脾腫を認めるが，胎児奇形を認めず（図 1）。

羊水：淡黄色，清明。CMV DNA 7.2×10^6 copy/ml。

胎児腹水：淡黄色，清明。CMV DNA 2.3×10^4 copy/ml。

臍帯静脈血：pH 7.326，BE 0.4 mmol/l，Hb 10.5 g/dl，CMV IgG 陽性（定性法）。

2. 確定診断

サイトメガロウイルスによる症候性胎内感染（ウイルスの再活性化による）と随伴する胎児水腫，胎児貧血。

3. 方針と治療

産婦人科，小児科，麻酔科との協議のうえで，ご夫婦，ご家族に数次にわたり治療方針を説明し，選択，同意を得たうえで，胎内治療を試みる方針となった。

妊娠 30 週，32 週，34 週で CMV 高力価（平均 167.0 EIA 価，×199 NT 倍）γグロブリンを胎児腹腔内に投与した。投与量は 2 g/推定体重 kg とした。胎盤循環系の血流は計算に加えないこととした。

セボフルレンによる全身麻酔下に胎児腹腔内を穿刺，2 台の超音波装置を用いて胎児循環機能をモニターしながら，腹水を約 50 ml 緩徐に吸引した後に，CMV 高力価γグロブリンを投与した。投与 5 分後に毎回臍帯静脈血を穿刺し，アシドーシスの有無，胎児血算，生化学検査を施行した。治療後は，胎児心拍モニターを