and placental tissue produce and secrete large amounts of M-CSF[4] and maternal serum M-CSF levels in normal pregnancies increase with gestational age.[4,11,12] We previously demonstrated that maternal blood M-CSF levels were significantly increased before the clinical manifestations of pre-eclampsia in normotensive women at 18 weeks of gestation who later develop pre-eclampsia.[13] As for complicated pregnancy, we have shown a significant increase in blood M-CSF levels not only in pre-eclampsia[14] but also in normotensive pregnancies complicated by intrauterine fetal growth restriction (IUGR)[15] compared with those in normal pregnancies. These reports suggest that M-CSF has various physiological effects on tissues at the maternal–fetal interface.

A large number of cytokines show high activity within amniotic fluid and their production at the maternal–fetal interface has been reported.[16,17]

A large amount of M-CSF was contained in amniotic fluid and we estimated that M-CSF in amniotic fluid has various functions as do many cytokines. Near term, the marked physiological change occurring in pregnant women is the onset of labor. Thus, we focused on the relationship between changes in the M-CSF level in amniotic fluid and labor induction.

On the basis of these findings, we focused on M-CSF levels in amniotic fluid and evaluated whether M-CSF levels in amniotic fluid during labor differed from those before the onset of labor in normal pregnancies. With respect to women during labor, we also examined whether M-CSF levels in amniotic fluid in the established labor group differed from those in advanced labor group.

SUBJECTS AND METHODS

Subjects

Informed consent was obtained from all subjects. The subjects were 48 normal pregnant women carrying single fetuses. Of these women, 24 were women during labor that resulted in subsequent delivery: 22 led to subsequent term delivery (labors); two had premature delivery. The gestational age of 22 labors ranged from 37.3 to 40.6 weeks of gestation. The other 24 were women before the onset of labor without uterine contractions who received cesarean section. Their gestational age ranged from 37.0 to 41.1 weeks of gestation. These 24 served as controls. These two groups (22 labors and 24 controls) were compared. Table I shows the characteristics of 46 subjects. Average gestational age at entry was 38 weeks of gestation. The women's ages, gestational ages and parity did not differ significantly between the two groups. The indications for cesarean section are shown in Table II. All cesarean sections were performed by spinal anesthesia.

Subjects who delivered small-for-gestational-age (SGA) infants were excluded from the study. SGA infants were defined as weighing less than the 10th percentile for gestational age from the standard growth curve for the Japanese,[18] and appropriate-for-gestational-age (AGA) infants were defined as having weights within normal ranges (10–90th percentile).

Subjects with any symptom of infection or abnormal C-reactive protein level were excluded from the study. Hyperglycemia was not detected in any subject under fasting conditions. There were no subjects with any comorbid condition such as diabetes, asthma, congenital heart disease, kidney disease, connective tissue disorders, and autoimmune disease. None of the subjects smoked tobacco. All neonates had normal anatomies.

Verification of Uterine Contractions

The presence or absence of uterine contractions was verified by cardiotocogram (CTG). The instrument used for CTG monitoring was MT-430 (Toitu, Tokyo, Japan). At admission, all patients in the third trimester of pregnancy and those with suspected uterine contractions underwent a CTG. All patients in labor were additionally monitored by CTG from 5 cm cervical dilatation to delivery.

TABLE I. The Characteristics of 46 Subjects

	Age (years)	Gestational age (weeks)	Parity	Systolic BP (mmHg)	Diastolic BP (mmHg)	*n*
With labor	31.1 ± 4.2	38.8 ± 0.8	(0) = 12;	116 ± 13.7	70 ± 13.7	22
(range)	(24–42)	(37.3–40.6)	(≥1) = 10	(94–138)	(32–88)	
Without labor	33.4 ± 3.9	38.3 ± 0.9	(0) = 7;	109 ± 12.0	65 ± 9.8	24
(range)	(24–40)	(37.1–41.1)	(≥1) = 17	(85–129)	(40–80)	
Significance	NS	NS	NS	NS	NS	

Data and results are mean ± S.D.
BP, blood pressure; NS, not significant.

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TABLE II. The Indication for Cesarean Section

	Indication for C-S	*n*
With labor	Repeated C-S	1
Without labor	Repeated C-S	14
	Breech presentation	4
	Previous myomectomy	3
	Placenta previa	2
	CPD	1

C-S, cesarean, section; CPD, cephalopelvic disproportion.

Selection of Women During Labor

We applied the following inclusion criteria to the selection of women during labor: (1) established labor group who were in active labor with regular uterine contractions at intervals of less than 10 min and progression of cervical dilatation on consecutive vaginal examination of 2–5 cm at the time of delivery; and (2) advanced labor group who were in active labor with cervical dilatation greater than 5 cm.

The established labor group and advanced labor group consisted of 10 and 12 women, respectively.

Amniotic Fluid Sampling

As for samples from women before the onset of labor, 24 samples of amniotic fluid were collected from the uterine cavity during cesarean section.

Samples from women during labor were collected at delivery: one sample from the uterine cavity during cesarean section; six from the vagina immediately after artificial rupture of membranes; 15 from the vagina immediately after spontaneous rupture of membranes.

When amniotic fluid flowed from the uterus into the vagina during labor in the absence of bleeding, we carefully collected only amniotic fluid using a syringe with an 18G-needle to avoid contamination by vaginal discharge.

Samples were centrifuged at 1600 × *g* for 10 min at room temperature. The separated supernatant was collected and stored at −30°C for subsequent determination of the M-CSF level.

Determination

The M-CSF level was determined by the sandwich enzyme-linked immunosorbent assay (ELISA) method with three antibodies: equine antihuman M-CSF antibody coated on a microtiter plate; an antibody solution containing highly purified rabbit antihuman M-CSF antibody; and caprine enzyme-conjugated antirabbit IgG antibody, according to the method reported by Hanamura et al.[19]

The % recovery of exogenous M-CSF mixed with sera collected from healthy volunteers was between 94 and 107%. Intra- and inter-assay coefficients of variation were 2.4 and 2.6% at concentrations of 632 U/mL and 2.1 and 1.8% at 1034 U/mL, respectively. The calculated M-CSF level in the serum gradually decreased as the serum concentration increased, proceeding in order from 20 to 40 to 60 to 80 to 100%. The M-CSF level calculated from data obtained from 100% serum added reached 75% of that obtained from 10% serum added. However, calculated M-CSF levels remained almost constant when the assay was carried out using more than fivefold diluted serum. This indicates that human serum samples should be diluted more than fivefold for the determination of M-CSF level to avoid interference from contaminating proteins. During determination, serum samples were diluted 11-fold in the present study.

Statistical Analysis

Parity was statistically analyzed by the chi-square test. M-CSF levels were statistically analyzed by Mann–Whitney *U*-test. All other data were statistically analyzed by the unpaired *t*-test. Significance was established at the $P < 0.05$ level.

RESULTS

Table III shows a summary of the results.

M-CSF Levels in Amniotic Fluid in Women with Labor and Those Without Labor

M-CSF levels in amniotic fluid were slightly higher in women with labor than in women without labor, but the difference was not significant. Fig. 1 is a scatter diagram with bar graphs for values of M-CSF in amniotic fluid. Although the M-CSF values were distributed across wide ranges: 1910–8690 U/mL in women with labor; 1910–10,400 U/mL in women

TABLE III. Determined Values of M-CSF in Amniotic Fluid, and Other Results in Normal Pregnancies

	M-CSF (U/mL)	Neonatal birth weight (g)	Placenta weight (g)	*n*
With labor (range)	4290 (1910–8690)	3004 ± 224 (2628–3530)	547 ± 76 (440–730)	22
Without labor (range)	3435 (1910–10400)	3068 ± 344 (2552–3680)	574 ± 100 (370–800)	24
Significance	NS ($P = 0.12$)	NS ($P = 0.46$)	NS ($P = 0.32$)	

M-CSF values are median (range). Other results are mean ± S.D. (range). NS, not significant.

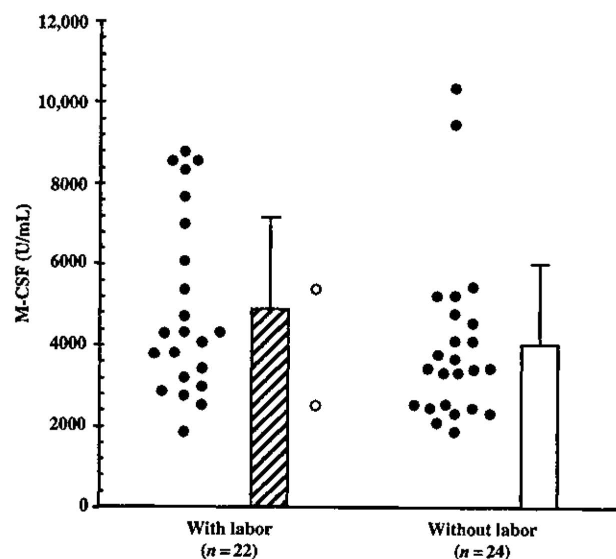

Fig. 1. A scatter diagram with bar graphs of M-CSF values in amniotic fluid in women with labor and those without labor. Bars indicate mean values and the vertical lines on the bars indicate S.D. M-CSF levels in amniotic fluid did not differ significantly between the two groups. In women with labor, closed circles indicate M-CSF values for 22 mothers who had term deliveries, and open circles indicate two mothers who had premature babies.

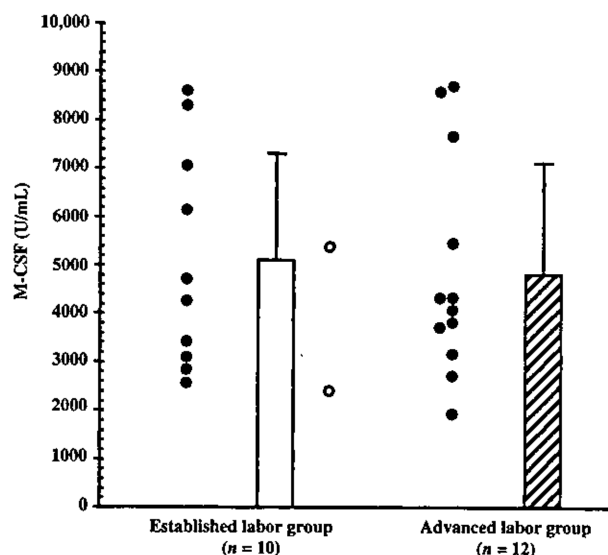

Fig. 2. A scatter diagram with bar graphs of M-CSF values in amniotic fluid in the established labor group and the advanced labor group. Bars indicate mean values and the vertical lines on the bars indicate S.D. Significant differences were not seen in M-CSF levels in amniotic fluid in the established labor group compared with those in the advanced labor group. In established labor group, closed circles indicate M-CSF values for 10 mothers who had term deliveries, and two open circles indicate two mothers who had premature babies.

without labor, these median values showed a concentration of 4290 U/mL in women with labor, and of 3435 U/mL in women without labor.

There were no pre-term deliveries among the women without labor. However, there were two patients who had babies prematurely due to pre-term labor among the women with labor; they delivered at 34.7 and 36.4 weeks of gestation and their M-CSF levels were 5390 and 2430 U/mL, respectively.

M-CSF Levels in Established Labor Group and Advanced Labor Group

Fig. 2 is a scatter diagram with bar graphs for values of M-CSF in amniotic fluid. The M-CSF values were distributed across wide ranges: 2510–8590 U/mL in the established labor group; 1910–8690 U/mL in the advanced labor group. M-CSF levels in amniotic fluid in the established labor group were 4485 U/mL (median; range, 2510–8590 U/mL) and those in the advanced labor group were 4180 U/mL (1910–8690 U/mL). The M-CSF levels were nearly the same between the two groups, and the difference was not significant ($P = 0.95$).

Neonatal Birth Weight

Neonatal birth weights were nearly the same between the women with labor and those without labor. There was no significant difference in neonatal birth weight between the two groups.

Placenta Weight

Placenta weights were nearly the same in women with labor as in women without labor, and the difference was not significant.

DISCUSSION

We evaluated M-CSF levels in amniotic fluid in women whose average gestational age at entry was 38 weeks of gestation. In the present study, discussing the volume and composition of amniotic fluid is beneficial. Amniotic fluid volume shows rather marked individual differences and increases with progression of pregnancy. From the time of amniotic sac formation, the amniotic fluid volume rapidly increases to the average peak volume of about 1000 mL at 36–38 weeks.[20,21] In some but not all pregnancies, the volume decreases as term approaches. There are many mechanisms that will account for all the variations in composition and volume of amniotic fluid. Amniotic fluid in early pregnancy is primarily produced by the amniotic membrane covering the placenta and cord. As pregnancy progresses, from about the fourth month, the fetus participates in modulating amniotic fluid composition and volume by urinating and swallowing progressively larger amounts of fluid. At the same time, movement of fluid into and out of the respiratory

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tract by fetal thoracic movements further modifies the volume and composition of the fluid.

In the mouse, M-CSF is released by maternal tissues immediately adjacent to the invasive trophoblast due to examination of maternal decidual tissue dissected from placentas at various stages of gestation.[22] In the human, the main sources of M-CSF in amniotic fluid are amniotic membranes, fetal urine, or maternal decidua adjacent to the maternal–fetal interface. The present study has the advantage of measuring M-CSF values in amniotic fluid contact with the maternal–fetal interface where immunological activity progressed.

We speculate that women with normotensive pregnancies complicated by IUGR and women who deliver SGA infants show high M-CSF levels in amniotic fluid because serum M-CSF levels are reported to be significantly higher in women who delivered SGA infants than in those who delivered AGA infants.[15] Thus, subjects who delivered SGA infants were excluded from the study.

If intrauterine infection, fetal distress, abruptio placentae, or uterine rupture was the indication for cesarean section, M-CSF level in amniotic fluid might be altered. The indications in this series were repeated cesarean section, breech presentation, previous myomectomy, placenta previa, or cephalopelvic disproportion. These might not influence M-CSF level in amniotic fluid.

Previous study has shown that there was a significant elevation of IL-18 levels in pregnant sera after the onset of labor and the increased levels persisted until at least the third day of puerperium.[23] Elevation of IL-18 in sera was also observed in various complicated pregnancies, such as premature rupture of the membranes (PROM), acute fatty liver of pregnancy and fetal growth restriction. IL-18 may have related to the onset of labor and/or labor may have contributed to IL-18 production.

Granulocyte colony-stimulating factor, IL-6 and IL-8 play an important role in modifying inflammatory reaction. According to another study,[24] these three inflammatory cytokines in amniotic fluid have shown a three- to fourfold increase during labor and an eight- to 13-fold increase in the presence of endotoxin. Since all three cytokines were simultaneously induced in amniotic fluid by labor and infection and there was a significant positive correlation among the three cytokine levels, labor and infection may have triggered the production of inflammatory cytokines at term and/or these cytokines may have contributed to the inflammatory events leading to labor.

Maymon et al. have shown that the levels of tumor necrosis factor-α (TNF-α) in amniotic fluid was significantly increased in patients in spontaneous term labor (median 6.4 pg/mL) compared those at term not in labor (4.1 pg/mL), while levels of TNF receptor 1 and TNF receptor 2 significantly decreased.[25] TNF-α and TNF-α soluble receptor profiles differed in term parturition.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine involved in reproduction. Ietta et al.[26] have shown that amniotic fluid MIF levels were significantly higher at term (median 62.10 ng/mL) than at midtrimester (20.07 ng/mL) and reached a peak during term labor (258.80 ng/mL). The elevated production and secretion of MIF in amniotic fluid at term, particularly during term labor, may have indicated that MIF contributed to the inflammatory events resulting in term labor.

There were not a few cytokines that are elevated in association with labor. The present study showed that M-CSF values in amniotic fluid at term did not increase during labor and were similarly distributed in women with labor compared with those without labor as shown in Fig. 1. It is very important to compare distribution of M-CSF values in amniotic fluid between women at the first stage of labor and those at the late stage because changes in M-CSF values between these stages clearly demonstrate the physiological role of M-CSF. The present study also showed that the distribution of M-CSF values in amniotic fluid at term were similar between the established labor group and the advanced labor group as shown in Fig. 2 when we divided the women during labor into the established labor group with 2–5 cm cervical dilatation and the advanced labor group with cervical dilatation greater than 5 cm. In addition to term delivery, we examined cases of premature delivery. M-CSF values for two mothers who had their babies prematurely at 34.7 and 36.4 weeks of gestation were distributed similarly compared with those who had term deliveries. These findings showed that there was no association between the presence of active labor and M-CSF levels in amniotic fluid in the absence of intrauterine infection.

Previous studies have shown that M-CSF plays a central role in maintaining the placenta and pregnancy.[27,28] Furthermore, we demonstrated significant increases in M-CSF levels[29] as well as GM-CSF levels[30] relative to total protein values in placental tissues in pre-eclampsia than in normal pregnancies. M-CSF is highly related to the maintenance of pregnancy and the pathophysiology of pre-eclampsia. However, M-CSF in amniotic fluid may not contribute to the onset of labor and/or labor leading to subsequent delivery may not induce the production and secretion of M-CSF into amniotic cavity.

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Medroxyprogesterone acetate attenuates estrogen-induced nitric oxide production in human umbilical vein endothelial cells☆

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Abstract

We report the novel observation that medroxyprogesterone acetate (MPA) attenuates the induction by 17β estradiol (E2) of both nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) activity in human umbilical vein endothelial cells. Although MPA had no effect on basal NO production or basal eNOS phosphorylation or activity, it attenuated the E2-induced NO production and eNOS phosphorylation and activity. Moreover, we examined the mechanism by which MPA attenuated the E2-induced NO production and eNOS phosphorylation. MPA attenuated the E2-induced phosphorylation of Akt, a kinase that phosphorylates eNOS. Treatment with pure progesterone receptor (PR) antagonist RU486 completely abolished the inhibitory effect of MPA on E2-induced Akt phosphorylation and eNOS phosphorylation. In addition, the effects of actinomycin D were tested to rule out the influence of genomic events mediated by nuclear PRs. Actinomycin D did not affect the inhibitory effect of MPA on E2-induced Akt phosphorylation. Furthermore, the potential roles of PRA and PRB were evaluated. In COS cells transfected with either PRA or PRB, MPA attenuated E2-induced Akt phosphorylation. These results indicate that MPA attenuated E2-induced NO production via an Akt cascade through PRA or PRB in a non-genomic manner.
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Keywords: Nitric oxide; eNOS; Medroxyprogesterone acetate; Endothelial cells; Akt; 17β estradiol; Progesterone receptor; RU486; Actinomycin D; Non-genomic events

The risk of cardiovascular disease steeply increases after menopause. Many epidemiological and basic studies have been shown that estrogen has the significant function in the vasculature of preventing the primary development of cardiovascular disease in women [1,2]. We and others have reported that estrogen acts directly on endothelial nitric oxide synthase (eNOS) activity through a non-genomic mechanism [3], resulting in rapid dilatation of blood vessels [4].

In the Women's Health Initiatives (WHI) large prospective randomized controlled study, although women on the conjugated equine estrogen (CEE)-medroxyprogesterone acetate (MPA) arm had an increase in the relative risk of cardiovascular events and breast cancer [5], the more recent reports on women on CEE-only treatment arm did not show increased cardiovascular disease [6]. Thus, there is a possibility that progestin has an adverse effect on the cardiovascular system. In fact,

☆ *Abbreviations:* MPA, medroxyprogesterone acetate; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; E2, 17β estradiol; PR, progesterone receptor; DAF-2,4,5,-diaminofluorescein.

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medroxyprogesterone acetate (MPA), commonly used as a progestine combined with estrogen, is reported to inhibit the endothelium-dependent vasodilatation induced by estrogen [7]. Thus, the mechanism by which progestin may interfere with the beneficial activities of estrogen should be determined. We therefore examined the effect of MPA on estrogen-induced eNOS activity and nitric oxide (NO) production in human umbilical vein endothelial cells (HUVECs). We report here the novel observation that MPA attenuates the induction by estrogen of both eNOS activity and NO production in HUVECs.

Materials and methods

Materials

17β-E2, medroxyprogesterone acetate, RU-486, and actinomycin D were purchased from Sigma Chemical (St. Louis, MO). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Rabbit polyclonal anti-phospho Akt, anti-Akt, anti-phospho eNOS (ser-1177), and anti-eNOS antibodies were obtained from New England BioLabs (Beverly, MA).

Methods

Cell culture. HUVECs were isolated according to the method of Jaffe et al. [8], plated in gelatin-coated tissue culture wells, and grown in M199 medium containing 20% fetal bovine serum and 50 μg/ml endothelial cell growth supplement (Clonetics Corp., San Diego, CA). HUVECs were used at passage 2 or 3. COS cells were cultured at 37 °C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a water-saturated atmosphere of 95% O_2 and 5% CO_2.

DNA transfection. COS cells were maintained in Dulbecco's modified Eagle's medium/2 mM glutamine (PAA Laboratories) supplemented with 10% fetal bovine serum (PAA Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin. COS cells cultured in 100-mm dishes were transfected with 1 μg ERα expression vector (HEGO), 0.5 μg HEGO + 0.5μg progesterone receptor A expression vector (HEGO + PR A), or 0.5μg HEGO + 0.5 μg progesterone receptor B expression vector (HEGO + PR B) using LipofectAMINE plus as described previously [9]. Twenty-four hours after transfection, the cells were placed under serum-deprived conditions, and then the cells were treated with various ligands. The cells were harvested and assayed for reporter activity or used for Western blot analysis. Each experiment was performed a minimum of three times, and a representative example is shown.

Measurement of NO production in living cells. NO was measured by use of DAF-2 (Daiichi Pure Chemicals, Tokyo, Japan), an NO-sensitive fluorescent dye [10]. HUVECs were grown to 95% confluence and were serum-starved for 24 h. Then the cells were loaded with 10 μM DAF-2 for 60 min at 37 °C. The DAF-2 fluorescence was measured by use of a fluorescence microscope camera with a filter set permitting excitation at 490 nm and emission at 515 nm. It has been reported that DAF-2 fluorescence increases almost linearly with the NO concentration. Therefore, we expressed the intracellular NO production as the net increment of DAF-2 fluorescence in 15 min relative to its basal value.

Assay of eNOS activity. HUVECs were cultured in 100-mm dishes. The cells were serum-starved overnight in phenol red-free medium before eNOS activity measurements. eNOS activity was determined as the conversion of radiolabeled L-arginine to L-citrulline by a method described previously [11,12] with a minor modification. Briefly, 10 μl of a sample was incubated for 10 min at 37 °C in a solution consisting of 50 mM Hepes, 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.1 mM tetrahydro-L-biopterin, 1 mM NADPH, 10 μg/ml calmodulin, 10 μM FAD, and 1.55 μM L-[guanidino-^{14}C]arginine (pH 7.8), in a final volume of 100 μl. The reaction was terminated by the addition of 200 μl buffer A (100 mM Hepes, 10 mM EDTA, pH 5.2). The whole reaction mixture was then applied to a 0.3-ml Dowex 50-WX column (Na^+ form, 200–400 mesh) that had been equilibrated with buffer A. [^{14}C]citrulline was eluted with 0.5 ml of buffer A, and then radioactivity was measured with a liquid scintillation counter. For activity assay, each data point was determined in triplicate and the average and standard deviation were calculated.

Western blot analysis. The cells were incubated in phenol red-free medium without serum for 24 h and then treated with various agents. They were then washed twice with phosphate-buffered saline and lysed in ice-cold HNTG buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) [13]. The lysates were centrifuged at 12,000g at 4 °C for 15 min, and the protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blocking was done in 10% bovine serum albumin in 1× Tris-buffered saline. Western blot analyses were performed with various specific primary antibodies. Immunoreacted bands in the immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system.

Statistics. Statistical analysis was performed using Student's t test, and $p < 0.01$ was considered significant. Data are expressed as means ± SE.

Results

MPA attenuates the E2-induced NO production and eNOS activity

NO production was examined by a fluorescence assay using an NO-sensitive dye, 4,5,-diaminofluorescein (DAF-2). HUVECs loaded with DAF-2 were treated with 10^{-7} M E2 for the indicated times. The increase in NO production induced by 10^{-7} M E2 reached a plateau at 15 min and rapidly declined thereafter (Fig. 1A). The cells were then treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA. Although MPA had no effect on the basal NO production, MPA significantly attenuated the E2-induced NO production (Fig. 1B).

We reported that E2 induces eNOS activity [3] which is critical in NO production in HUVECs. Therefore, we evaluated the effect of MPA on E2-induced eNOS activity. HUVECs were treated with 10^{-7} M E2 for the indicated times, and then the eNOS activity was examined by measuring the conversion of arginine to citrulline. As we reported previously [3], the increase in eNOS activity induced by 10^{-7} M E2 reached a plateau at 10 min and rapidly declined thereafter (Fig. 1C). The cells were then treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA. 10^{-7} M MPA significantly attenuated the E2-induced eNOS activity (Fig. 1D).

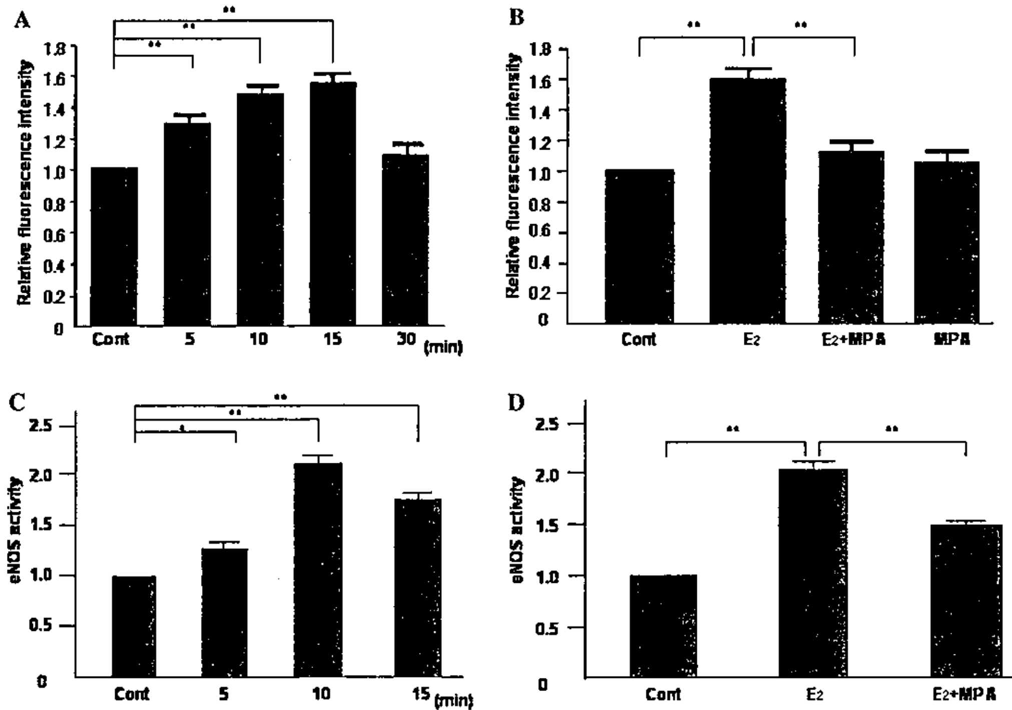

Fig. 1. MPA attenuates the E2-induced NO release and eNOS activation. To examine NO release, HUVECs were loaded with DAF-2 and then treated with 1 nM E2 for the indicated times (A), or treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min (B). To examine eNOS activity, HUVECs were treated with 1 nM E2 for the indicated times (C), or treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min (D). eNOS activity was measured by the conversion of L-[guanidino-^{14}C] arginine to L-[guanidino-^{14}C]citrulline, as described under Materials and methods. The basal activity of eNOS was arbitrarily set at 1.0. Data are expressed as the mean-fold activation ± SE of three separate experiments. Significant differences are indicated by asterisks. $*p < 0.05$; $**p < 0.01$.

MPA attenuates the E2-induced eNOS phosphorylation and Akt phosphorylation

eNOS activity is regulated by its phosphorylation via the PI3K-Akt cascade, and we reported that E2 induced eNOS phosphorylation via the PI3K-Akt cascade in HUVECs [3]. Therefore, we first evaluated the effect of MPA on E2-induced eNOS phosphorylation. Cells were treated with E2 for various times and then used to prepare lysates that were subjected to Western blotting using anti-phospho-eNOS or -eNOS antibody. Although E2 did not affect the expression of eNOS (Fig. 2A,

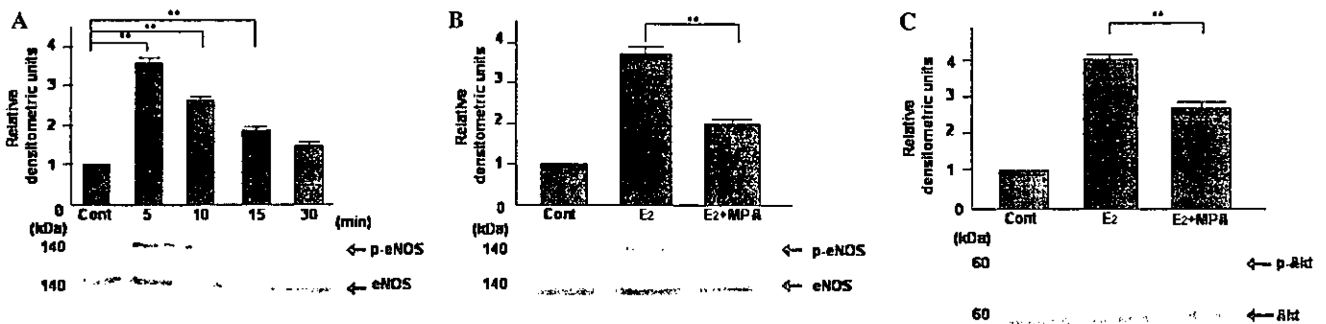

Fig. 2. MPA attenuates the E2-induced eNOS phosphorylation and Akt phosphorylation. HUVECs were treated with 1 nM E2 for the indicated times (A) or treated with 1 nM E2, 1 nM E2 + 10^{-6} M MPA for 15 min (B) and then harvested and used to prepare cell lysates. The lysates were subjected to Western blotting using anti-phospho-eNOS (middle panels) or anti-eNOS (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-eNOS bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. (C) HUVECs were treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min and then harvested and used to prepare cell lysates. The lysates were subjected to Western blotting using anti-phospho-Akt (middle panels) or anti-Akt (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-Akt bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. The values shown represent means ± SE from at least three separate experiments. Significant differences are indicated by asterisks. $**p < 0.01$.

lower panel), it significantly induced the phosphorylation of eNOS (Fig. 2A, middle and top panels). Cells were treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA. Although 10^{-7} M MPA had no effect on the basal eNOS phosphorylation, it significantly attenuated the E2-induced eNOS phosphorylation (Fig. 2B).

We next evaluated the effect of MPA on E2–induced Akt phosphorylation. Cells were treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA and then used to prepare lysates that were subjected to Western blotting using anti-phospho-Akt or -Akt antibody. Although 10^{-7} M MPA had no effect on the basal Akt phosphorylation, it significantly attenuated the E2-induced Akt phosphorylation (Fig. 2C).

Involvement of non-genomic PR activation

We further examined the mechanism by which MPA attenuated the E2-induced eNOS phosphorylation. To determine if this response involves rapid progesterone receptor (PR) activation, the effect of concomitant treatment with the pure PR antagonist RU486 was determined. RU486 completely abolished the inhibitory effects of MPA on E2-induced eNOS phosphorylation (Fig. 3A) and Akt phosphorylation (Fig. 3B).

Moreover, the effects of actinomycin D, an inhibitor of gene transcription, were tested to rule out the influence of genomic events mediated by nuclear PRs. Actinomycin D did not affect the inhibition by MPA of E2-induced Akt phosphorylation (Fig. 3C), indicating that the effects of MPA were independent of gene transcription regulation, and are thus termed "non-genomic." These results suggest that MPA attenuated the E2-induced eNOS phosphorylation and Akt phosphorylation via non-genomic PR activation.

Effect of PRA or PRB expression on the inhibitory effect of MPA on E2-induced Akt phosphorylation

We also evaluated the potential role of PRA or PRB in the inhibitory effect of MPA on E2-induced Akt phosphorylation. We confirmed that both PRA and PRB were expressed in HUVECs (data not shown). Therefore, COS cells, which do not express PRA or PRB, were used to examine which of these receptors is involved in the inhibitory effect of MPA on E2-induced

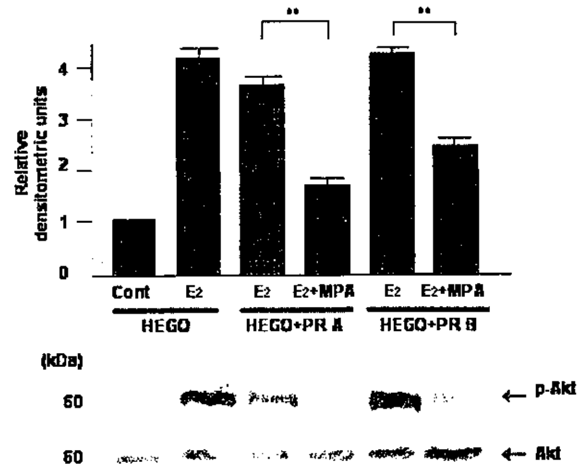

Fig. 4. Effect of PRA or PRB expression on the inhibitory effect of MPA on E2-induced Akt phosphorylation. COS cells were transfected with 1 μg wild-type estrogen receptor vector (HEGO) (lanes 1 and 2), and 0.5 μg progesterone receptor A vector and 0.5 μg HEGO (lanes 3 and 4), 0.5 μg progesterone receptor B vector and 0.5 μg HEGO (lanes 5 and 6). Then, transfected COS cells were treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min. Akt phosphorylation was measured as described in the legend for Fig. 2. Relative densitometric units of the bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. Values shown represent means ± SE from at least three separate experiments. Significant differences are indicated by asterisks. $^{**}p < 0.01$.

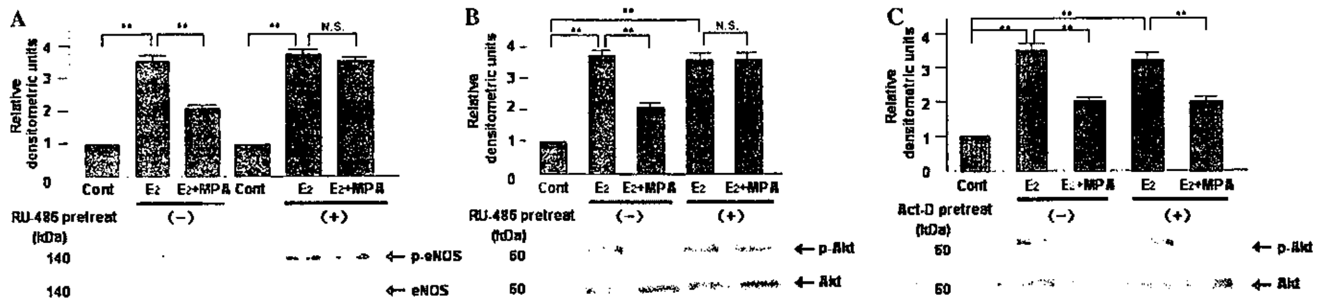

Fig. 3. Involvement of non-genomic PR. HUVECs were pre-incubated with or without 1 nM RU-486 for 1 h and then treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min and then harvested and used to prepare cell lysates. The lysates were subjected to Western blotting using anti-phospho-eNOS (A, middle panels), anti-eNOS (A, bottom panels), anti-phospho-Akt (B, middle panels), or anti-Akt (B, bottom panels) antibody (A). (C) HUVECs were pre-incubated with or without 25 μg/ml actinomycin D (Act-D) for 120 min and then treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min. The lysates were subjected to Western blotting using anti-phospho-Akt (middle panels) or anti-Akt (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. Each experiment was repeated three times with essentially identical results. Significant differences are indicated by asterisks. $^{**}p < 0.01$.

Akt phosphorylation. In COS cells transfected with either PRA or PRB, MPA attenuated the E2-induced Akt phosphorylation (Fig. 4). These results indicate that MPA attenuated the E2-induced Akt phosphorylation through PRA or PRB.

Discussion

There are several mechanisms through which estrogen exerts cardio-protective effects [14]. In the WHI study, although women on the CEE-only treatment arm did not have an increase in the relative risks of cardiovascular events [6], women on the CEE-MPA arm had an increase in the relative risks of cardiovascular events [5]. These findings of the WHI study suggest the possibility that progestin has an adverse effect on the cardiovascular system. Does MPA have adverse effects on all of the cardio-protective functions of estrogen? In this report, we demonstrated that MPA attenuates the E2-induced NO production and eNOS activity in HUVECs, providing a molecular mechanism to account for the clinical findings that MPA inhibits the endothelium-dependent vasodilatation by estrogen [7]. Although it remains possible that MPA has adverse effects on cardio-protective functions of estrogen other than NO production and eNOS activation, MPA at least had no effect on estrogen-induced cell proliferation of HUVECs (data not shown).

How does MPA attenuate the E2-induced NO release and eNOS activity? It was reported that eNOS is one of the substrates of Akt [15] and that the activity of eNOS is regulated by its phosphorylation via the PI3K-Akt cascade [16]. In this report, we showed that MPA attenuates the E2-induced eNOS phosphorylation and Akt phosphorylation. Moreover, actinomycin D did not affect the inhibitory effects of MPA on E2-induced eNOS phosphorylation and Akt phosphorylation, indicating the involvement of a non-genomic response of PR, as reported previously [17–19].

There are two isoforms of PRs, PRA, and PRB. What is the different role of PRA and PRB in the biological actions of progestin? It was reported that an imbalance in the native ratio of the two isoforms can lead to alterations in PR signaling [20] and mammary gland development [21]. In this report, we demonstrated that both PRA and PRB are involved in the MPA-induced attenuation of E2-induced Akt phosphorylation. It was reported that PRB is required for the induction by MPA of cyclin D1 expression via the ERK cascade in MCF-7 cells [22]. Thus, the effects of MPA on the signaling cascades are different depending on the tissues. Although the involvement of non-genomic PRA and PRB in inhibiting E2-induced Akt phosphorylation is a novel finding, further investigations will be necessary to fully clarify the molecular mechanism of the adverse effect of MPA on the cardiovascular system.

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

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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

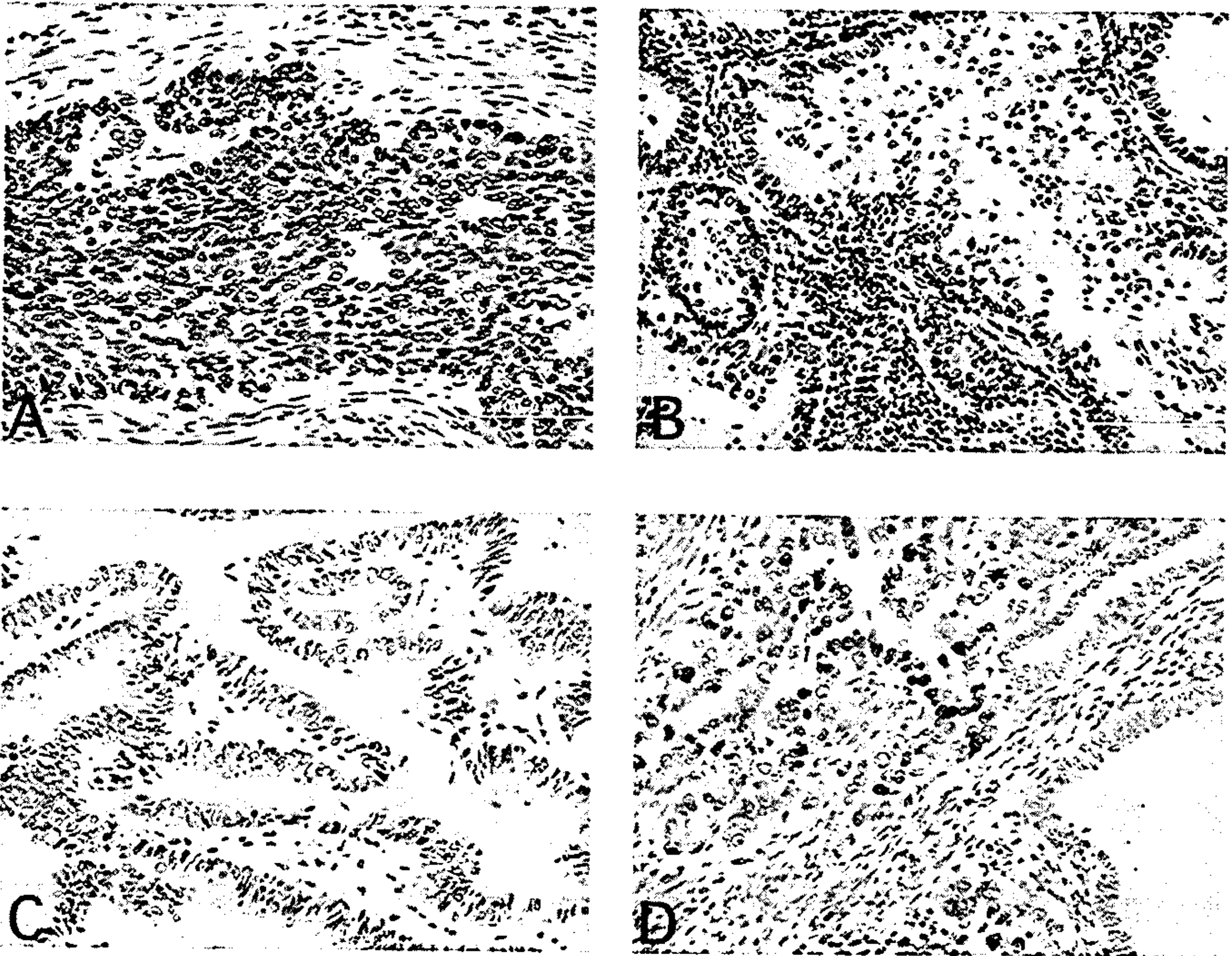

Figure 1. PTEN or p53 expression in endometrial carcinoma. (A) Abundant expression of PTEN. (B) Loss of PTEN expression. (C) Reduced PTEN expression. These three cases represent grade 3 (G3) endometrioid adenocarcinoma (A), G2 endometrioid adenocarcinoma (B), and G1 endometrioid adenocarcinoma (C). (D) 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

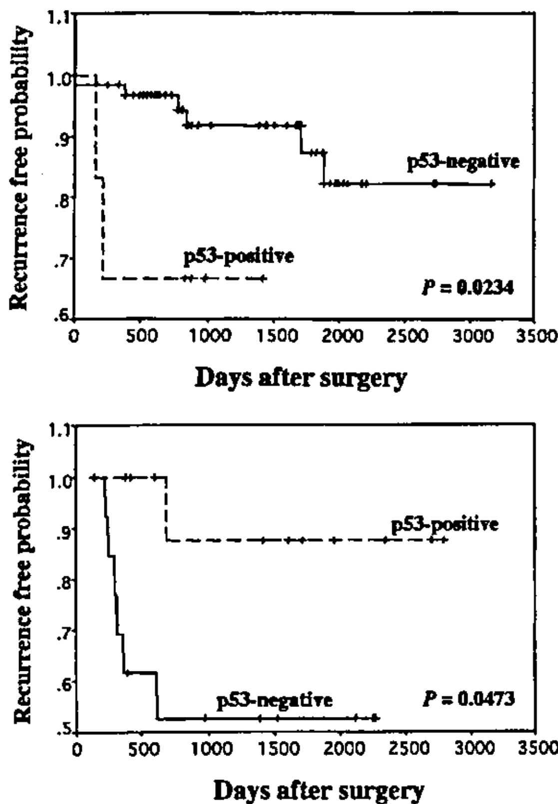

Figure 2. (A) Recurrence-free probability (RFP) for patients who had G1 and G2 endometrioid adenocarcinoma (G1 and G2 group) with or without a p53 abnormality. (B) RFP for patients who had G3 endometrioid adenocarcinoma and other phenotypes (G3 and others group) with or without a p53 abnormality.

(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.

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