研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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V. 研究成果の刊行物・別刷

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Lactation and Risk of Endometrial Cancer in Japan: A Case-Control Study

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Okamura, C., Tsubono, Y., Ito, K., Niikura, H., Takano, T., Nagase, S., Yoshinaga, K., Terada, Y., Murakami, T., Sato, S., Aoki, D., Jobo, T., Okamura, K. and Yaegashi, N. *Lactation and Risk of Endometrial Cancer in Japan: A Case-Control Study.* Tohoku J. Exp. Med., 2006, **208** (2), 109-115 —— The incidence of endometrial cancer is rapidly increasing in Japan. Although the risk factors in European populations have been well described, there are few epidemiologic studies regarding risk factors for endometrial cancer in Japanese women. This hospital-based case-control study among Japanese women was carried out from 1998 to 2000. The cases were selected from women with endometrial cancer (n =155), and the controls selected from women attending the university gynecological outpatient clinic for cervical cancer screening (n = 96). Subjects were interviewed to ascertain breast feeding practices, contraceptive usage, as well as potential risk factors for endometrial cancer. We observed a lower risk of endometrial cancer associated with oral contraceptive (OC) and a higher risk associated with higher body mass index (BMI), and older ages at first and last delivery. Gravidity reduced odds ratio (OR) for endometerial cancer to 0.34 (95% confidence interval [CI] 0.13-0.92). Compared with parous women who had never breastfed, the multivariate OR for women with a history of breastfeeding was 0.37 (95% CI, 0.17-0.82). Additionally, a greater lapse of time since breastfeeding increased OR for endometrial cancer by over three times. In conclusion, the present study has indicated that breastfeeding reduces the risk of endometrial cancer in Japanese women.

——— endometrial cancer; breastfeeding; risk factor; case-control study

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Age-adjusted incidence rates for endometrial cancer have doubled during the past two decades among Japanese women. The rising incidence possibly may be due to changes in lifestyle, or changes in reproductive factors such as childbearing and contraception, as these characteristics have been associated with endometrial cancer risk in Western populations. In Western countries, there is considerable evidence that reproductive factors play a role in the etiology of endometrial cancer. Nulliparity and obesity have been associated with a higher risk, whereas oral contraceptive (OC) use has been associated with a lower risk (Kirschner et al. 1981; Kelsey et al. 1982; Zumoff 1982; Austin et al. 1991; Schapira et al. 1991; Brinton et al. 1992; Shu et al. 1992; Kalandidi et al. 1996; McPherson et al. 1996; Iemura et al. 2000; Herrinton et al. 2001). A few studies have examined the association between breastfeeding and endometrial cancer risk (Rosenblatt and Thomas 1995; Salazar-Martinez et al. 1999; Newcomb and Trentham-Dietz 2000); however, the findings from these studies are inconsistent.

The reproductive characteristics of Japanese women, however, are different from those of Western populations. For instance, 15%, 36%, and 59% of contraceptive-using women choose OCs in the United States, France, and Germany, respectively, whereas the prevalence of OC use is only 1.5% among Japanese women who use contraception. Only 1.8% of Japanese women older than 50 years have used hormone replacement therapy (HRT), whereas the prevalence of HRT usage is 53% among US women aged 50-59 years. These differences make it difficult to generalize findings obtained in Western studies to Japanese women. There have, however, been a few studies evaluating risk factors for endometrial cancer in Japanese women (Inoue et al. 1994; Hirose et al. 1996, 1999). Therefore, this study was undertaken to further characterize endometrial cancer risk factors in Japanese population.

Subjects and Methods

This case-control study was a collaborative investigation in three areas of Japan (Tokyo, Kanagawa, and Miyagi). Cases were accrued from three university hospitals from January 1, 1998, through December 31, 2000. Eligible cases included Japanese women between 20 and 80 years of age who underwent surgery for a diagnosis of endometrioid endometrial cancer confirmed by histology. The cases resided in defined geographic catchment areas, and had not received treatment previously. One hundred sixty seven cases were eligible for the study and 12 subjects refused to participate. Thus, 155 (93%) of the eligible cases participated. Stage distribution of the cases was as follows: stage I, $n = 104$; stage II, $n = 14$; stage III, $n = 33$; and stage IV, $n = 4$.

The controls were selected from women who attended gynecologic outpatient clinics in the university hospitals for cervical cancer screening. Controls included only women with intact uteri. Ninety six women were included as controls; however, 9 women refused participation (participation rate, 91%). Cases and controls were not matched in terms of age or other variables.

The protocol for this study was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan).

Gynecologists interviewed the cases and controls using a standard questionnaire asking about demographic information, medical history, cigarette use, and reproductive history (parity, gravidity, and ages at first pregnancy, last delivery, menarche, menopause, and lactation). Body mass index (BMI) was calculated based on self-reports of weight (kg)/height $(m)^2$. The distribution of continuous variables was examined among cases and controls and divided into two or three categories.

To estimate the risk of endometrial cancer associated with various factors, we calculated age-adjusted and multivariate odds ratio (ORs) along with 95% confidence interval (CI) using unconditional logistic regression analysis. Statistical Analysis System (SAS Institute, Cary, NC, USA) software was used for all statistical analyses.

Results

The mean ages of cases and controls were 56.1 years and 49.6 years, respectively. Table 1 presents age-adjusted ORs and 95% CIs of the selected variables for the risk of endometrial cancer. Higher BMI was associated with higher risk ($p = 0.01$). OC use was associated with a lower risk of disease (OR, 0.16; 95% CI, 0.04-0.66), although only three cases and ten controls used OCs. Intra-uterine device use, history of HRT, smoking, sterility, hypertension, diabetes mellitus,

TABLE 1. *Baseline characteristics of cases and controls*

Caracteristics	Cases	%	Controls	%	Age-adjusted OR	95% CI	*p* value
Age (years)							
< 45	15	9.7	39	40.6			
45-55	52	33.6	23	24			
55-65	55	35.4	24	25			
≧ 65	33	21.3	10	10.4			
BMI (kg/m^2)							
< 20.04	36	23.3	26	27.1	1.00		
20.04-21.63	27	17.4	35	36.5	0.47	0.22-0.99	
21-64-23.92	45	29.0	20	20.8	1.24	0.58-2.67	
≧ 23.93	47	30.3	15	15.6	1.92	0.86-4.30	0.01
Oral contraceptive use							
Never	152	98.1	86	89.6	1.00		
Ever	3	1.9	10	10.4	0.16	0.04-0.66	0.01
IUD use							
Never	148	95.5	90	93.8	1.00		
Ever	7	4.5	6	6.2	0.54	0.17-1.71	0.29
HRT use							
Never	132	85.16	85	88.5	1.00		
Ever	23	14.84	11	11.5	1.4	0.63-3.14	0.41
Cigarette smoking							
Never	126	81.3	77	80.2	1.00		
Ever	29	18.7	19	19.8	1.30	0.65-2.61	0.52
Sterility							
Never	143	92.3	87	90.6	1.00		
Ever	12	7.7	9	9.4	0.81	0.31-2.11	0.66
Hypertension							
Never	115	74.2	87	90.6	1.00		
Ever	40	25.8	9	9.4	2.15	0.95-4.86	0.45
Diabetes mellitus							
Never	139	89.7	92	95.8	1.00		
Ever	16	10.3	4	4.2	1.82	0.56-5.92	0.32
Personal cancer history							
Never	139	89.7	92	96.8	1.00		
Ever	16	10.3	4	4.2	1.78	0.55-5.73	0.33

and personal cancer history were not associated with risk. There were 20 persons who had personal cancer history. Among them 11 persons had breast cancer and the remaining nine persons had cancer history at various sites, such as colon cancer, rectal cancer, thyroid cancer, gastric cancer, lung cancer, and ovarian cancer. Four of the 20 persons had hormone therapy.

Table 2 shows the ORs for the association of endometrial cancer with reproductive factors.

TABLE 2. *Multivariate Odds Ratio and 95%Confidence Intervals for Endometrial Cancers-According to Reproductive Factors*

Variables	Cases	%	Controls	%	OR*	95% CI	*p* value
Menopausal status							
Pre	51	32.9	55	57.3	1.00		
Post	104	67.1	41	42.7	0.91	0.39-2.14	0.82
Gravidity							
Never	20	12.9	9	9.4	1.00		
Ever	135	87.1	87	90.6	0.34	0.13-0.92	0.03
No. of pregnancies							
0	20	12.9	9	9.4	1.00		
1	27	17.4	16	16.7	0.43	0.14-1.33	
2	42	27.1	32	33.3	0.34	0.12-0.97	
≧ 3	66	42.6	39	40.6	0.29	0.10-0.85	0.04
Parity							
Never	36	23.2	21	21.9	1.00		
Ever	119	76.8	75	78.1	0.46	0.22-0.96	0.04
No. of deliveries							
0	36	23.2	21	21.9	1.00		
1	29	18.7	18	18.8	0.45	0.18-1.12	
2	68	43.9	44	45.8	0.47	0.21-1.04	
≧ 3	22	14.2	13	13.5	0.44	0.16-1.20	0.1
Age at first delivery**							
≦ 24	43	36	11	14.7	1.00		
25-26	36	30.3	23	30.7	0.45	0.18-1.10	
27-29	21	17.7	23	30.7	0.30	0.12-0.78	
≧ 30	19	16	18	24	0.35	0.13-0.96	0.05
Age at last delivery**							
≦ 25	23	19.3	6	8	1.00		
26-30	40	33.6	25	33.3	0.48	0.16-1.45	
31-33	39	32.8	26	34.7	0.45	0.15-1.36	
≧ 34	17	14.3	18	24	0.28	0.08-0.94	0.02

* OR adjusted for age, BMI, and oral contraceptive use.

** Parous women only.

The ORs were adjusted for age, BMI, and OC use. Gravidity was inversely associated with endometrial cancer risk. Women who reported ever being pregnant had only one third the risk of endometrial cancer compared with women who had never been pregnant (OR, 0.34; 95% CI, 0.13-0.92, $p = 0.03$). Women who reported three or more pregnancies had about one third the risk of women with no pregnancies (OR, 0.29; 95% CI, 0.10-0.85).

Parity was also inversely associated with endometrial cancer risk. Women who reported ever having delivery had about one half the risk of endometrial cancer compared with women who had never delivered (OR, 0.46; 95% CI, 0.22-0.96, $p = 0.04$). Higher age at the first or last deliveries was associated with a lower risk for endometrial cancer ($p = 0.05$, $p = 0.02$). Age at menarche, menopausal status, age at menopause, history of dysmenorrhea, and history of abortion were not associated with risk (data not shown).

Only parous women, representing 119 cases and 75 controls, were included in the analysis of the association between breastfeeding and endometrial cancer risk presented in Table 3. Table 3 also showed the age distribution of both cases and control and that of the lapse of the last breast feeding. The ORs were adjusted for age, BMI, and OC use as shown in Table 3. Compared with parous women who had never breastfed, the multivariate odds ratio for women who had ever breastfed was 0.37 (95% CI: 0.17-0.82, $p = 0.013$). A greater lapse of time since breastfeeding concluded was directly associated with an increased risk of endometrial cancer (OR of 20-29 years, 3.10, 95% CI: 1.14-8.48, and OR of 30 or longer, 3.85, 95% CI: 1.00-14.84, $p = 0.045$). Then, we analyzed the association between frequency or duration of breastfeeding and endometrial cancer risk, but did not find any significant association (data not shown).

Discussion

In this hospital-based case-control study among Japanese women, we observed a lower risk of endometrial cancer associated with OC use and gravidity, and a higher risk associated with higher BMI, older ages at first and last delivery and number of pregnancies. These findings were consistent with data obtained in prior Japanese studies (Inoue et al. 1994; Hirose et al. 1996, 1999). In contrast to the study by Inoue et al. (1994), our study failed to demonstrate an association between a history of hypertension, diabetes mellitus, or cancer.

Our study also demonstrated a reduction in the risk of endometrial cancer associated with breastfeeding. The proportion of never breastfeeding (35.3%) in endometrial cancer cases was larger than that in control, but the risk was signifi-

Table 3. *Multivariate Odds Ratio for Endometrial Cancers in relation to breastfeeding and age among parous women*

Variables	Cases (*n*)					Controls (*n*)					OR*	95% CI	*p* value
	Total	< 45	45-55	55-65	65 ≦	Total	< 45	45-55	55-65	65 ≦			
breastfeeding													
Never	42	1	10	24	7	11	3	3	2	3	1.00		
Ever	77	1	31	26	19	64	25	14	18	7	0.37	0.17-0.82	0.013
Years since last breastfed**													
1-19	12	1	9	1	1	33	24	7	2	0	1.00		
20-29	31	0	17	12	2	20	1	7	11	1	3.10	1.14-8.48	
≧ 30	34	0	5	13	16	11	0	0	5	6	3.85	1.00-14.84	0.045

* Adjusted for age, BMI and oral contraceptive use.

** Ever breastfed women only.

cant even after been adjusted for age, BMI, and contraceptive use. The risk reduction of endometrial cancer was associated not only with breastfeeding itself but also with time since the last breastfeeding. From 1982 to 2000, seven case-control studies conducted in six countries, including four developing countries, examined the relationship between breastfeeding and the risk of endometrial cancer. Four early studies, two of which were Japanese, failed to support an association (Kelsey et al. 1982; Brinton et al. 1992; Hirose et al. 1996, 1999). Three recent Western studies, however, suggested a protective effect of breastfeeding (Rosenblatt and Thomas 1995; Salazar-Martinez et al. 1999; Newcomb and Trentham-Dietz 2000). This effect was more pronounced with recent breastfeeding, diminishing as the history of breastfeeding became more remote (Rosenblatt and Thomas 1995; Newcomb and Trentham-Dietz 2000). Our findings were consistent with those of the latter studies, making this the first report that notes an inverse association between breastfeeding and the risk of endometrial cancer among Japanese women.

Exposure of the endometrium to estrogen in the absence of progesterone is thought to increase the risk of endometrial cancer (Key and Pike 1988- see comment for citation). In lactating women, the ovarian cycle is suppressed and blood estrogen levels are reduced (Baird et al. 1979). In the case of oral contraceptives, progesterone continually opposes estrogen, minimizing the duration of time the endometrium is exposed to unopposed estrogen. Thus, suppression of circulating estrogen levels, or opposition of estrogen by progesterone, may represent a biological mechanism accounting for the protective effects of pregnancy, oral contraceptives, and breastfeeding against carcinogenesis of endometrial tissue.

Among Japanese women, the birth rate decreased 28.1 to 9.3 per 1,000 during 1950-2000, and the proportion of women who exclusively breastfed decreased from 70.5% to 44.8% during the same period (Kaneda 2003). In our study, the proportion of women who breastfed for 13 months was 52.4%. The observed lower risk associated with breastfeeding in this study suggests that the recent increase in incidence of endometrial cancer in Japan may be in part attributed to a decrease in both the number of pregnancies and the prevalence of breastfeeding.

A limitation of this study was its lack of age matching. This resulted in a mean age of cases that was 6 years older than that of controls. It is unlikely that the lack of age matching resulted in serious distortion of our observations because all analyses were adjusted for continuous age. Furthermore, the findings in these studies were consistent with data obtained in several previous studies. Another limitation of the study was the small number of control. To overcome these limitations, in progress is our new case control study which matched ages of cases and controls and included two times more subjects of control. These data will confirm the present observations.

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Expression of retinoic acid receptors in human endometrial carcinoma

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The retinoids (vitamin A and its biologically active derivatives) are essential for the health and survival of the individual. Several studies have reported a strong rationale for the use of retinoids in cancer treatment and chemoprevention. It has been discovered that expression of retinoic acid receptor (RAR) β is frequently silenced in epithelial carcinogenesis, which has led to the hypothesis that RARβ could act as a tumor suppressor. However, the status of RARβ in human endometrial carcinoma has not been examined. In the present study, we initially studied the effects of retinoic acid on cell proliferation and the expression of RARα, RARβ, and RARγ using AM580 (a RAR-specific agonist) in the Ishikawa endometrial cancer cell line. We also examined the expression of RAR in human eutopic endometrium (30 cases), endometrial hyperplasia (28 cases), and endometrial carcinoma (103 cases) using immunohistochemistry. Finally, we correlated these findings with the clinicopathological parameters. *In vitro*, cell growth was inhibited and RARβ and RARγ mRNA was significantly induced by AM580, compared with vehicle controls, whereas RARα mRNA was significantly attenuated by AM580, compared with vehicle. RARβ was detected predominantly in endometrial hyperplasia, compared with endometrial carcinoma. No statistically significant correlation was obtained between the expression of any other RAR subtypes and clinicopathological parameters in human endometrial carcinoma. The results of our study demonstrate that AM580 inhibits cell growth and induces RARβ mRNA expression in the Ishikawa cell line, and the expression level of RARβ in endometrial carcinoma is significantly lower than that in endometrial hyperplasia. AM580 might therefore be considered as a potential treatment for endometrial carcinoma. (*Cancer Sci* 2008; 99: 267–271)

The retinoic acids are natural and synthetic derivatives of vitamin A that regulate a variety of important cellular functions. A strong rationale exists for the use of retinoids in cancer therapy and chemoprevention based on preclinical, epidemiological, and clinical findings.[1–3]

All-*trans*-retinoic acid (ATRA) activates the classical nuclear retinoic acid receptors (RAR), whereas 9-*cis*-retinoic acid activates the RAR and non-classical nuclear retinoid X receptors (RXR).[4,5] There are six genes encoding retinoid receptors: *RARα*, *RARβ*, and *RARγ*, as well as *RXRα*, *RXRβ*, and *RXRγ*. Multiple receptor isoforms exist through the alternate usage of splice sites and promoters. The ligand-binding domains of RAR and RXR are distinct, and can be pharmacologically targeted individually. RAR can heterodimerize with RXR, and RXR can heterodimerize with other nuclear receptors, including the thyroid hormone receptors, vitamin D receptor, and peroxisomal proliferator activated receptor.[5]

Endometrial carcinoma is one of the most common female pelvic malignancies in the world, and its incidence has increased recently.[6] Although little is known about the molecular events involved in the pathogenesis, a close relationship has been observed between estrogenic stimulation of the endometrium and the appearance of endometrial hyperplasia.[7] Studies in experimental animals have shown that retinoids, particularly ATRA, may play an important role in regulating the effects of estrogens on the endometrium. Studies with vitamin A-deficient rats demonstrated that physiological levels of retinoic acids suppress endometrial hyperplasia and metaplasia associated with chronic estrogen administration.[8] In immature ovariectomized rats, pharmacological doses of retinoic acids suppressed estrogen-induced endometrial stromal-cell proliferation.[9]

In the normal human endometrium, intracellular retinoic acid concentrations in both epithelial and stromal cells are elevated during the secretory phase.[10] Kumarendran *et al.* reported the expression of RARα, RARβ, RARγ, and RXRα mRNA in human eutopic endometrium using northern blotting.[11] Siddiqui *et al.* also reported the presence of RAR and RXR mRNA in endometrioid endometrial carcinoma using northern blotting.[12] However, the details of the status of these retinoid receptors and the correlation between retinoid receptors and clinical outcomes have not been studied in normal and diseased human endometrium.

In the present study, we initially examined the effects of retinoic acid on cell proliferation and the expression of RARα, RARβ, and RARγ using AM580 (a synthetic RAR-specific ligand) in the Ishikawa endometrial cancer cell line. We then examined the expression of RAR in human eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma using immunohistochemistry. Finally, we correlated these findings with clinicopathological parameters in endometrial carcinoma.

Materials and Methods

Cell culture. The human endometrial cancer cell line Ishikawa was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco/BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (growth medium). Fresh suspensions of stromal cells were plated in culture dishes and incubated at 5% CO_2 and 37°C. Media was changed at 72-h intervals until the cells became 70–80% confluent. Confluent cells were serum-deprived for 16 h in serum-free, phenol red-free DMEM/F-12 before being subjected to the following treatments for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and real-time reverse transcription (RT)-polymerase chain reaction (PCR): MTT assay, serum-free and phenol red-free medium with ethanol as the baseline control, or serum-containing, phenol red-free medium with AM580 (10^{-8}–10^{-6} M) for 24, 48, 72, and 96 h; real-time RT-PCR, serum-free and phenol red-free medium with ethanol as the baseline control, or serum-free and phenol red-free medium with AM580 (0.1 μM) for 1, 3, and 6 h.

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MTT cell-proliferation assay. Cell proliferation was assessed by a colorimetric assay using MTT. The MTT assay that detects the formation of dark-blue formazan produced from MTT in active mitochondria was carried out as reported previously.[13] At 4 h before the end of each experiment, 10 μL MTT solution was added into each well of 96-well plates. The optical absorbance at 570 nm was read within 30 min with a microplate reader (Thermomax, Molecular Devices, Menlo Park, CA, USA). Data are expressed in optical density units. The last column of each 96-well plate did not contain cells and was used as a blank.

Isolation of total RNA and real-time RT-PCR. Total RNA was isolated from the Ishikawa cells using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA). The concentration and quality of total RNA were determined spectrophotometrically and by electrophoresis on denaturing formaldehyde–agarose gels.

Reverse transcription reactions were carried out using the SUPERSCRIPT III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The expression levels of mRNA for RARα, RARβ, and RARγ were measured by real-time RT-PCR using a standard TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and probes were obtained from the ABI TaqMan Gene Expression Assay catalog (Applied Biosystems). The probes contained a 6-carboxy-fluorescein phosphoramidite (FAM dye) label at the 5′ end and a minor groove binder and non-fluorescent quencher at the 3′ end, and were designed to hybridize across exon junctions. For each sample, triplicates were run for each gene in a 384-well format plate. Template cDNA and TaqMan Gene Expression Assays, which contain PCR primers and probes, were added to TaqMan Universal PCR Mastermix (Applied Biosystems) to a final volume of 20 μL. The reactions were incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (CT) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. TaqMan CT values were converted into absolute copy numbers. All RNA samples were normalized based on the TaqMan Gene Expression Assays for the human glyceraldehyde-3-phosphate dehydrogenase endogenous control (Applied Biosystems).

Tissue preparation. Thirty cases of eutopic endometrium, 28 cases of endometrial hyperplasia, and 103 cases of endometrial endometrioid adenocarcinoma (well-differentiated, 49 cases; moderately differentiated, 32 cases; poorly differentiated, 22 cases) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. This study was approved by the Ethical Committee of the Tohoku University School of Medicine. We obtained non-pathological endometria from hysterectomy specimens carried out due to carcinoma *in situ* of the uterine cervix. Endometrial hyperplasia and carcinoma specimens were obtained from total dilatation and curretage, and hysterectomy, respectively. None of the patients had undergone irradiation or chemotherapy before surgery. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization and staged according to the International Federation of Gynecology and Obstetrics system.[14] All specimens were processed routinely (i.e. 10% formalin fixed for 24–48 h), paraffin embedded, and thin sectioned (3 μm).

Immunohistochemistry. Polyclonal antibodies for RARα (sc-551), RARβ (sc-552), and RARγ (sc-550) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunohistochemical analysis was carried out using the streptavidin–biotin amplification method with a Histofine kit (Nichirei, Tokyo, Japan), which has been described previously in detail.[15] The dilutions of the primary antibodies used in our study were as follows: RARα, 1/500; RARβ, 1/5; and RARγ, 1/500. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2), and counterstained with hematoxylin. Tissue sections of skin were used as positive controls for RARα and RARγ and those of human breast cancer were used for RARβ.

Scoring of immunohistochemistry. Evaluation of RAR was carried out in high-power fields (×400) using a standard light microscope. Two of the authors (K. T. and M. T.) simultaneously searched the entire tissue sections and determined the most representative areas using a double-headed microscope. For evaluation of immunoreactivity of the RAR, we determined the labeling index (LI; the percentage of positive cells) according to the report by Sasano and colleagues.[15] After completely reviewing the immunostained sections of each lesion, a total of more than 500 tumor cells from three different representative fields were counted independently. Cases with discordant results (interobserver differences of more than 5%) were reevaluated simultaneously by the same authors using a double-headed light microscope. Interobserver differences were less than 5% in the present study.

Statistical analysis. The statistical significance of the relationship between immunoreactivity for the RAR and clinical stage, histological grade, myometrial invasion, vascular involvement, recurrence rate, and overall survival were evaluated using the *t*-test. *P*-values less than 0.05 were considered significant.

Results

Effect of AM580 on Ishikawa cell proliferation. The proliferative effects of AM580 (10^{-8}–10^{-6} M) on Ishikawa cells were assessed using the MTT colorimetric assay. Ishikawa cells were treated with AM580 for 24–96 h. Following treatment for 24 h, there were no significant differences between the groups. However, at 48 h and thereafter, both of the AM580 groups (10^{-7} M and 10^{-6} M) had significantly decreased proliferation compared with the control group ($P < 0.05$; Fig. 1).

Effects of retinoic acid on the expression of RAR. To examine the effects of the RAR-specific ligand AM580 on the expression of RAR, Ishikawa cells were cultured for 1, 3, and 6 h with 0.1 μM

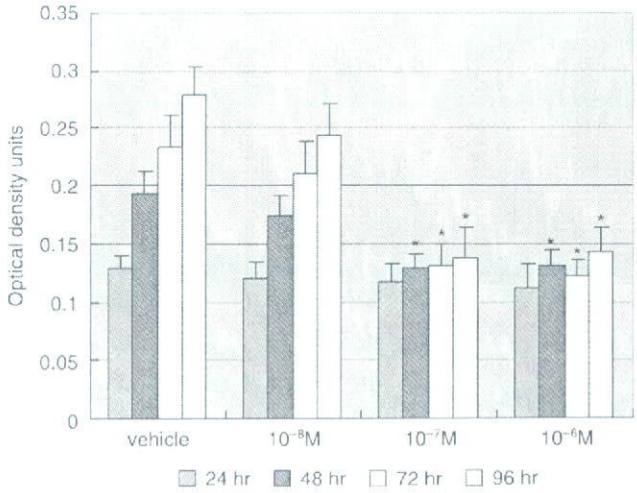

Fig. 1. Effect of AM580 on Ishikawa cell proliferation. Cells were treated with AM580 (10^{-8}–10^{-6} M) or with dimethylsulfoxide (DMSO; vehicle), as a control, for 24–96 h. Cell proliferation was analyzed in 96-well microplates by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Values are expressed as mean ± SEM of eight wells for each group. Following 24 h of treatment, there were no significant differences between the groups. However, at 48 h and thereafter, both of the AM580 groups (10^{-7} M and 10^{-6} M) had significantly decreased proliferation compared with the control group. *$P < 0.05$ versus vehicle.

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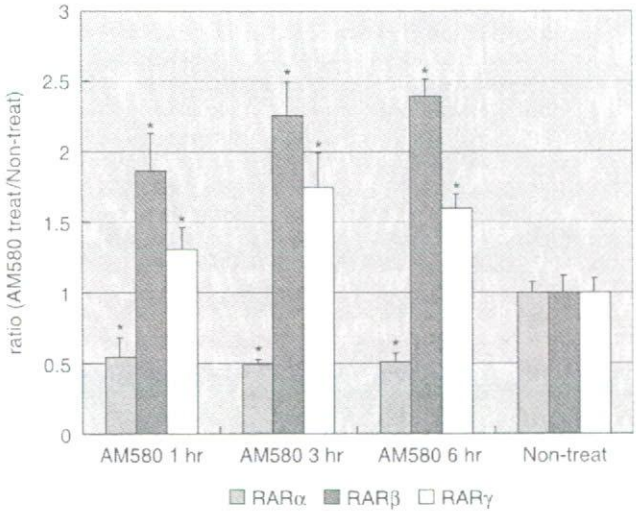

Fig. 2. Retinoic acid receptor (RAR) β and RARγ mRNA were induced by AM580 in Ishikawa cells. Real-time reverse transcription–polymerase chain reaction analysis was carried out to measure RAR mRNA expression in the presence or absence of AM580 treatment. Summary data for three independent experiments are shown. Results are expressed as the mean ± SE. $*P < 0.001$ versus non-treated.

AM580, and compared with vehicle-treated cells as controls. Real-time RT-PCR analysis was carried out to measure the mRNA expression of the respective RAR, in the presence or absence of AM580 treatment. As shown in Figure 2, RARβ and RARγ mRNA were induced significantly by AM580, compared with vehicle control ($P < 0.001$), whereas RARα mRNA was attenuated significantly by AM580, compared with vehicle ($P < 0.001$).

Cellular localization of RAR in eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. Immunohistochemistry for the RAR was carried out using serial sections of eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma (Fig. 3).

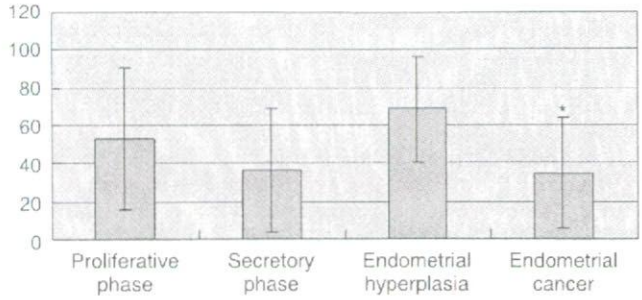

Fig. 4. Summary of immunohistochemistry for retinoic acid receptor (RAR) β. For evaluation of RARβ expression, we determined the labeling index (LI). Results are expressed as the mean ± SE. $*P < 0.02$ versus endometrial hyperplasia.

In ectopic endometrium, RARβ immunoreactivity was detected in the nuclei of 25–50% of stromal cells, whereas RARα immunoreactivity was present in the nuclei of 3–5% of stromal cells throughout the phases of the menstrual cycle. RARγ immunoreactivity was not detected in any of the epithelial cells examined. RARα and RARβ immunoreactivities were detected in the nuclei of epithelial cells throughout all menstrual phases.

In endometrial hyperplasia, immunoreactivity for RAR was detected in the nuclei of both epithelial and stromal cells. RARβ immunoreactivity was detected in the nuclei of 60–75% of stromal cells, whereas RARα and RARγ immunoreactivities were present in the nuclei of 10–15% of stromal cells.

In endometrial carcinoma, no RAR immunoreactivity was detected in the stromal cells in any of the cases examined. RARα and RARβ immunoreactivities were detected in the nuclei of 18–37% of carcinoma cells.

Relationship between the expression of RAR subtypes and clinicopathological parameters in endometrial carcinoma. As shown in Figure 4, RARβ was detected predominantly in endometrial hyperplasia, compared with endometrial carcinoma ($P = 0.014$; LI in endometrial hyperplasia 68.39 ± 28.31 vs LI in endometrial carcinoma 34.67 ± 28.95). There was no significant correlation between the RARβ

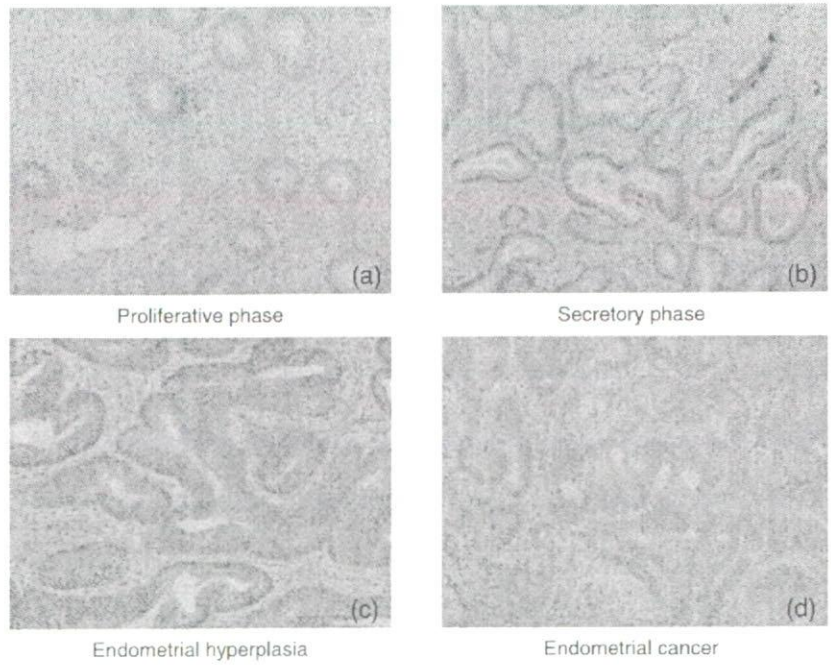

Fig. 3. Retinoic acid receptor (RAR) β localization in eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. (a,b) In ectopic endometrium, RARβ immunoreactivities were detected in the nuclei of epithelial cells throughout all menstrual phases. (c) In endometrial hyperplasia, RARβ immunoreactivity was detected in the nuclei of 60–75% of stromal cells. In endometrial carcinoma, no RARβ immunoreactivity was detected in the stromal cells in any of the cases examined. (d) RARβ immunoreactivities were detected in the nuclei of 18–37% of carcinoma cells. Immunohistochemistry for the RAR was carried out using serial sections of eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. (Original magnification, ×200.)

Table 1. Summary of the relationship between retinoic acid receptor (RAR) expression and clinicopathological findings in endometrial cancer

Clinicopathological characteristic (number of patients)	RAR			*P*-value
	α	β	γ	
Stage				
I (66)	18.11 ± 10.06	36.62 ± 29.80	3.45 ± 3.17	NS
II (12)	22.33 ± 19.05	25.92 ± 30.19	3.25 ± 3.93	
III (22)	16.91 ± 11.18	33.50 ± 26.44	5.77 ± 8.69	
IV (3)	17.67 ± 5.51	35.33 ± 30.62	3.67 ± 2.08	
Grade				
Well-differentiated (49)	17.59 ± 9.62	36.27 ± 28.78	3.88 ± 3.42	NS
Moderate-differentiated (32)	22.19 ± 14.97	37.22 ± 30.36	3.28 ± 3.08	
Poorly differentiated (22)	14.36 ± 7.72	27.41 ± 27.33	5.00 ± 8.78	
Myometrial invasion				
<1/2 (62)	18.34 ± 11.74	34.08 ± 30.49	3.25 ± 2.98	NS
≥1/2 (38)	17.76 ± 11.42	33.05 ± 27.30	3.92 ± 3.35	
Vessel involvement				
+ (29)	17.62 ± 12.32	31.55 ± 29.74	3.83 ± 3.14	NS
– (34)	16.47 ± 6.72	35.38 ± 30.72	3.03 ± 2.94	
Recurrence				
+ (16)	15.19 ± 8.48	26.31 ± 26.27	3.06 ± 2.82	NS
– (87)	18.91 ± 11.93	36.21 ± 29.30	4.09 ± 5.26	
Prognosis				
Alive (95)	18.52 ± 11.57	34.14 ± 29.31	9.00 ± 14.04	NS
Dead (8)	16.13 ± 11.15	41.00 ± 25.07	3.51 ± 3.11	

For evaluation of RARs' expression, we determined the labeling index. Results are expressed as the mean ± SE. NS, not significant.

expression of each histological type. There was no significant correlation between the expression of each of the RAR subtypes in endometrial carcinoma (data not shown).

The relationships between the expression of the RAR subtypes and the clinicopathological findings in endometrial carcinoma are summarized in Table 1. There was no statistically significant correlation between LI for any of the other RAR subtypes and the clinicopathological parameters, including clinical stage, histological grade, myometrial invasion, vascular involvement, recurrence rate, and overall survival.

Discussion

Retinoic acids exhibit diverse biological properties that may potentially contribute to their antitumor effect. They inhibit cell proliferation and angiogenesis, and can induce cell differentiation and apoptosis.[16,17] RARβ repression has been reported in preneoplastic oral-cavity lesions,[18] non-small-cell lung cancer,[19–21] breast cancer,[22] and esophageal cancer.[23] Although other retinoid receptors were expressed in these tissues, only RARβ levels were significantly lower in the premalignant and tumor tissues. RARβ expression was selectively lost in premalignant oral lesions, and was able to be restored by retinoic acid treatment.[18] The restoration of RARβ expression was associated with a clinical response, suggesting a role for RARβ, both as a mediator of the retinoic acid response and as a biological marker in chemoprevention trials.[18] This was confirmed in renal cancer, in which upregulation of RARβ correlated with a response to 13-*cis*-retinoic acid and interferon α-2a.[24] Thus, the correlation with RARβ repression led to the hypothesis that RARβ could act as a tumor suppressor. In addition, introduction of RAR-β protein into retinoic acid-insensitive breast cancer cell lines has been shown to restore retinoic acid responsiveness.[25] In our study, RARβ was detected predominantly in endometrial hyperplasia, compared with endometrial carcinoma. These results suggest that suppression of RAR-β expression may inhibit the differentiation of endometrial epithelium in endometrial carcinoma.

In recent studies, the retinoid isotretinoin was not effective for chemoprevention in stage I non-small-cell lung cancer or early stage head and neck squamous-cell carcinoma.[26,27] The retinoid-signaling pathway was studied in normal and neoplastic tissues to determine why preclinical retinoid activity did not readily translate into clinical success. It was discovered that expression of RARβ is frequently silenced in epithelial carcinogenesis, which led to the hypothesis that RARβ acts as a tumor suppressor that is partially responsible for the limited clinical activity of classical retinoids.[28,29] To examine the effect of the RAR-specific ligand AM580 on RARβ expression, we carried out MTT assay and real-time RT-PCR analysis using the Ishikawa cell line. Although AM580 inhibited cell growth and induced RARβ mRNA expression in Ishikawa cells, no statistically significant correlation was obtained between the expression of RARβ and clinicopathological parameters in human endometrial carcinoma. RARβ has four isoforms that are generated differentially by means of the promoters P1 and P2 and alternative splicing.[30] Our studies evaluated RARβ expression as a monolithic entity and did not distinguish between the various RARβ isoforms that have been identified in humans. Differential expression of different RARβ isoforms, at least in part, might underlie the contradictory associations of RARβ expression. However, it awaits further investigations for clarification.

Retinoids are useful tools for identifying critical target genes and pathways that can reduce carcinogenesis.[31,32] Accumulating evidence suggests that retinoids play a role in regulating the function of the endometrium.[33,34] Retinoids have also been reported to affect the expression of a number of genes in the endometrium, such as matrix metalloproteinases and interleukin-6.[35] Although the profile of retinoid receptors of epithelial cells has been elucidated,[11,12,36] the effect of retinoids on the proliferation of normal epithelial cells remains unknown. In our study, AM580 inhibited cell growth and induced RARβ mRNA expression in Ishikawa cells, and the expression level of RARβ in endometrial carcinoma was significantly lower than that in endometrial hyperplasia. AM580 might possibly be used as a treatment for

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endometrial carcinoma. However, it awaits further investigations for clarification.

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Expression of retinoic acid receptors in human endometrial carcinoma

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The retinoids (vitamin A and its biologically active derivatives) are essential for the health and survival of the individual. Several studies have reported a strong rationale for the use of retinoids in cancer treatment and chemoprevention. It has been discovered that expression of retinoic acid receptor (RAR) β is frequently silenced in epithelial carcinogenesis, which has led to the hypothesis that RARβ could act as a tumor suppressor. However, the status of RARβ in human endometrial carcinoma has not been examined. In the present study, we initially studied the effects of retinoic acid on cell proliferation and the expression of RARα, RARβ, and RARγ using AM580 (a RAR-specific agonist) in the Ishikawa endometrial cancer cell line. We also examined the expression of RAR in human eutopic endometrium (30 cases), endometrial hyperplasia (28 cases), and endometrial carcinoma (103 cases) using immunohistochemistry. Finally, we correlated these findings with the clinicopathological parameters. *In vitro*, cell growth was inhibited and RARβ and RARγ mRNA was significantly induced by AM580, compared with vehicle controls, whereas RARα mRNA was significantly attenuated by AM580, compared with vehicle. RARβ was detected predominantly in endometrial hyperplasia, compared with endometrial carcinoma. No statistically significant correlation was obtained between the expression of any other RAR subtypes and clinicopathological parameters in human endometrial carcinoma. The results of our study demonstrate that AM580 inhibits cell growth and induces RARβ mRNA expression in the Ishikawa cell line, and the expression level of RARβ in endometrial carcinoma is significantly lower than that in endometrial hyperplasia. AM580 might therefore be considered as a potential treatment for endometrial carcinoma. (*Cancer Sci* 2008; 99: 267–271)

The retinoic acids are natural and synthetic derivatives of vitamin A that regulate a variety of important cellular functions. A strong rationale exists for the use of retinoids in cancer therapy and chemoprevention based on preclinical, epidemiological, and clinical findings.[1–3]

All-*trans*-retinoic acid (ATRA) activates the classical nuclear retinoic acid receptors (RAR), whereas 9-*cis*-retinoic acid activates the RAR and non-classical nuclear retinoid X receptors (RXR).[4,5] There are six genes encoding retinoid receptors: *RARα*, *RARβ*, and *RARγ*, as well as *RXRα*, *RXRβ*, and *RXRγ*. Multiple receptor isoforms exist through the alternate usage of splice sites and promoters. The ligand-binding domains of RAR and RXR are distinct, and can be pharmacologically targeted individually. RAR can heterodimerize with RXR, and RXR can heterodimerize with other nuclear receptors, including the thyroid hormone receptors, vitamin D receptor, and peroxisomal proliferator activated receptor.[5]

Endometrial carcinoma is one of the most common female pelvic malignancies in the world, and its incidence has increased recently.[6] Although little is known about the molecular events involved in the pathogenesis, a close relationship has been observed between estrogenic stimulation of the endometrium and the appearance of endometrial hyperplasia.[7] Studies in experimental animals have shown that retinoids, particularly ATRA, may play an important role in regulating the effects of estrogens on the endometrium. Studies with vitamin A-deficient rats demonstrated that physiological levels of retinoic acids suppress endometrial hyperplasia and metaplasia associated with chronic estrogen administration.[8] In immature ovariectomized rats, pharmacological doses of retinoic acids suppressed estrogen-induced endometrial stromal-cell proliferation.[9]

In the normal human endometrium, intracellular retinoic acid concentrations in both epithelial and stromal cells are elevated during the secretory phase.[10] Kumarendran *et al.* reported the expression of RARα, RARβ, RARγ, and RXRα mRNA in human eutopic endometrium using northern blotting.[11] Siddiqui *et al.* also reported the presence of RAR and RXR mRNA in endometrioid endometrial carcinoma using northern blotting.[12] However, the details of the status of these retinoid receptors and the correlation between retinoid receptors and clinical outcomes have not been studied in normal and diseased human endometrium.

In the present study, we initially examined the effects of retinoic acid on cell proliferation and the expression of RARα, RARβ, and RARγ using AM580 (a synthetic RAR-specific ligand) in the Ishikawa endometrial cancer cell line. We then examined the expression of RAR in human eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma using immunohistochemistry. Finally, we correlated these findings with clinicopathological parameters in endometrial carcinoma.

Materials and Methods

Cell culture. The human endometrial cancer cell line Ishikawa was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco/BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (growth medium). Fresh suspensions of stromal cells were plated in culture dishes and incubated at 5% CO_2 and 37°C. Media was changed at 72-h intervals until the cells became 70–80% confluent. Confluent cells were serum-deprived for 16 h in serum-free, phenol red-free DMEM/F-12 before being subjected to the following treatments for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and real-time reverse transcription (RT)-polymerase chain reaction (PCR): MTT assay, serum-free and phenol red-free medium with ethanol as the baseline control, or serum-containing, phenol red-free medium with AM580 (10^{-5}–10^{-6} M) for 24, 48, 72, and 96 h; real-time RT-PCR, serum-free and phenol red-free medium with ethanol as the baseline control, or serum-free and phenol red-free medium with AM580 (0.1 μM) for 1, 3, and 6 h.

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MTT cell-proliferation assay. Cell proliferation was assessed by a colorimetric assay using MTT. The MTT assay that detects the formation of dark-blue formazan produced from MTT in active mitochondria was carried out as reported previously.[13] At 4 h before the end of each experiment, 10 μL MTT solution was added into each well of 96-well plates. The optical absorbance at 570 nm was read within 30 min with a microplate reader (Thermomax, Molecular Devices, Menlo Park, CA, USA). Data are expressed in optical density units. The last column of each 96-well plate did not contain cells and was used as a blank.

Isolation of total RNA and real-time RT-PCR. Total RNA was isolated from the Ishikawa cells using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA). The concentration and quality of total RNA were determined spectrophotometrically and by electrophoresis on denaturing formaldehyde–agarose gels.

Reverse transcription reactions were carried out using the SUPERSCRIPT III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The expression levels of mRNA for RARα, RARβ, and RARγ were measured by real-time RT-PCR using a standard TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and probes were obtained from the ABI TaqMan Gene Expression Assay catalog (Applied Biosystems). The probes contained a 6-carboxy-fluorescein phosphoramidite (FAM dye) label at the 5′ end and a minor groove binder and non-fluorescent quencher at the 3′ end, and were designed to hybridize across exon junctions. For each sample, triplicates were run for each gene in a 384-well format plate. Template cDNA and TaqMan Gene Expression Assays, which contain PCR primers and probes, were added to TaqMan Universal PCR Mastermix (Applied Biosystems) to a final volume of 20 μL. The reactions were incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (CT) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. TaqMan CT values were converted into absolute copy numbers. All RNA samples were normalized based on the TaqMan Gene Expression Assays for the human glyceraldehyde-3-phosphate dehydrogenase endogenous control (Applied Biosystems).

Tissue preparation. Thirty cases of eutopic endometrium, 28 cases of endometrial hyperplasia, and 103 cases of endometrial endometrioid adenocarcinoma (well-differentiated, 49 cases; moderately differentiated, 32 cases; poorly differentiated, 22 cases) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. This study was approved by the Ethical Committee of the Tohoku University School of Medicine. We obtained non-pathological endometria from hysterectomy specimens carried out due to carcinoma *in situ* of the uterine cervix. Endometrial hyperplasia and carcinoma specimens were obtained from total dilatation and curretage, and hysterectomy, respectively. None of the patients had undergone irradiation or chemotherapy before surgery. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization and staged according to the International Federation of Gynecology and Obstetrics system.[14] All specimens were processed routinely (i.e. 10% formalin fixed for 24–48 h), paraffin embedded, and thin sectioned (3 μm).

Immunohistochemistry. Polyclonal antibodies for RARα (sc-551), RARβ (sc-552), and RARγ (sc-550) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunohistochemical analysis was carried out using the streptavidin–biotin amplification method with a Histofine kit (Nichirei, Tokyo, Japan), which has been described previously in detail.[15] The dilutions of the primary antibodies used in our study were as follows: RARα, 1/500; RARβ, 1/5; and RARγ, 1/500. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2), and counterstained with hematoxylin. Tissue sections of skin were used as positive controls for RARα and RARγ and those of human breast cancer were used for RARβ.

Scoring of immunohistochemistry. Evaluation of RAR was carried out in high-power fields (×400) using a standard light microscope. Two of the authors (K. T. and M. T.) simultaneously searched the entire tissue sections and determined the most representative areas using a double-headed microscope. For evaluation of immunoreactivity of the RAR, we determined the labeling index (LI; the percentage of positive cells) according to the report by Sasano and colleagues.[15] After completely reviewing the immunostained sections of each lesion, a total of more than 500 tumor cells from three different representative fields were counted independently. Cases with discordant results (interobserver differences of more than 5%) were reevaluated simultaneously by the same authors using a double-headed light microscope. Interobserver differences were less than 5% in the present study.

Statistical analysis. The statistical significance of the relationship between immunoreactivity for the RAR and clinical stage, histological grade, myometrial invasion, vascular involvement, recurrence rate, and overall survival were evaluated using the *t*-test. *P*-values less than 0.05 were considered significant.

Results

Effect of AM580 on Ishikawa cell proliferation. The proliferative effects of AM580 (10^{-5}–10^{-6} M) on Ishikawa cells were assessed using the MTT colorimetric assay. Ishikawa cells were treated with AM580 for 24–96 h. Following treatment for 24 h, there were no significant differences between the groups. However, at 48 h and thereafter, both of the AM580 groups (10^{-7} M and 10^{-6} M) had significantly decreased proliferation compared with the control group ($P < 0.05$; Fig. 1).

Effects of retinoic acid on the expression of RAR. To examine the effects of the RAR-specific ligand AM580 on the expression of RAR, Ishikawa cells were cultured for 1, 3, and 6 h with 0.1 μM

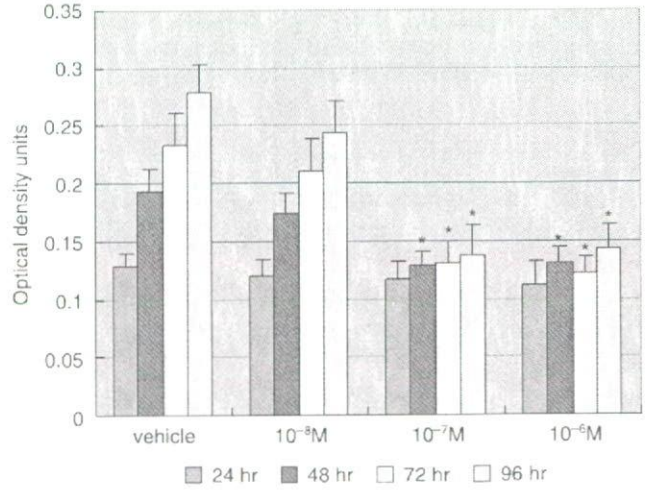

Fig. 1. Effect of AM580 on Ishikawa cell proliferation. Cells were treated with AM580 (10^{-5}–10^{-6} M) or with dimethylsulfoxide (DMSO; vehicle), as a control, for 24–96 h. Cell proliferation was analyzed in 96-well microplates by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Values are expressed as mean ± SEM of eight wells for each group. Following 24 h of treatment, there were no significant differences between the groups. However, at 48 h and thereafter, both of the AM580 groups (10^{-7} M and 10^{-6} M) had significantly decreased proliferation compared with the control group. *$P < 0.05$ versus vehicle.

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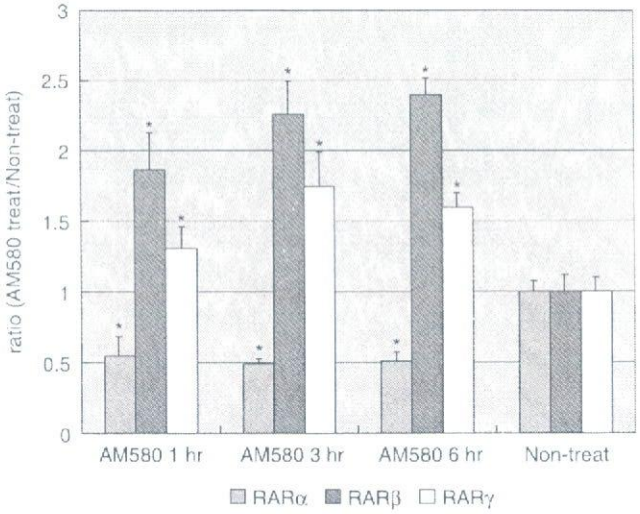

Fig. 2. Retinoic acid receptor (RAR) β and RARγ mRNA were induced by AM580 in Ishikawa cells. Real-time reverse transcription–polymerase chain reaction analysis was carried out to measure RAR mRNA expression in the presence or absence of AM580 treatment. Summary data for three independent experiments are shown. Results are expressed as the mean ± SE. *$P < 0.001$ versus non-treated.

AM580, and compared with vehicle-treated cells as controls. Real-time RT-PCR analysis was carried out to measure the mRNA expression of the respective RAR, in the presence or absence of AM580 treatment. As shown in Figure 2, RARβ and RARγ mRNA were induced significantly by AM580, compared with vehicle control ($P < 0.001$), whereas RARα mRNA was attenuated significantly by AM580, compared with vehicle ($P < 0.001$).

Cellular localization of RAR in eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. Immunohistochemistry for the RAR was carried out using serial sections of eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma (Fig. 3).

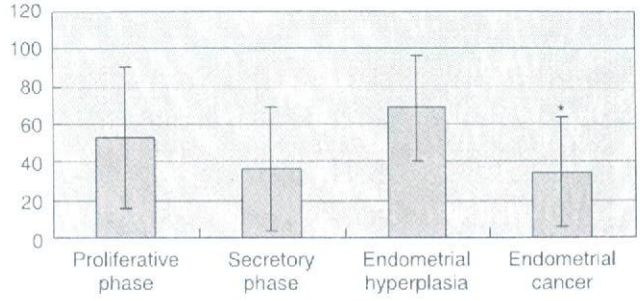

Fig. 4. Summary of immunohistochemistry for retinoic acid receptor (RAR) β. For evaluation of RARβ expression, we determined the labeling index (LI). Results are expressed as the mean ± SE. *$P < 0.02$ versus endometrial hyperplasia.

In ectopic endometrium, RARβ immunoreactivity was detected in the nuclei of 25–50% of stromal cells, whereas RARα immunoreactivity was present in the nuclei of 3–5% of stromal cells throughout the phases of the menstrual cycle. RARγ immunoreactivity was not detected in any of the epithelial cells examined. RARα and RARβ immunoreactivities were detected in the nuclei of epithelial cells throughout all menstrual phases.

In endometrial hyperplasia, immunoreactivity for RAR was detected in the nuclei of both epithelial and stromal cells. RARβ immunoreactivity was detected in the nuclei of 60–75% of stromal cells, whereas RARα and RARγ immunoreactivities were present in the nuclei of 10–15% of stromal cells.

In endometrial carcinoma, no RAR immunoreactivity was detected in the stromal cells in any of the cases examined. RARα and RARβ immunoreactivities were detected in the nuclei of 18–37% of carcinoma cells.

Relationship between the expression of RAR subtypes and clinicopathological parameters in endometrial carcinoma. As shown in Figure 4, RARβ was detected predominantly in endometrial hyperplasia, compared with endometrial carcinoma ($P = 0.014$; LI in endometrial hyperplasia 68.39 ± 28.31 vs LI in endometrial carcinoma 34.67 ± 28.95). There was no significant correlation between the RARβ

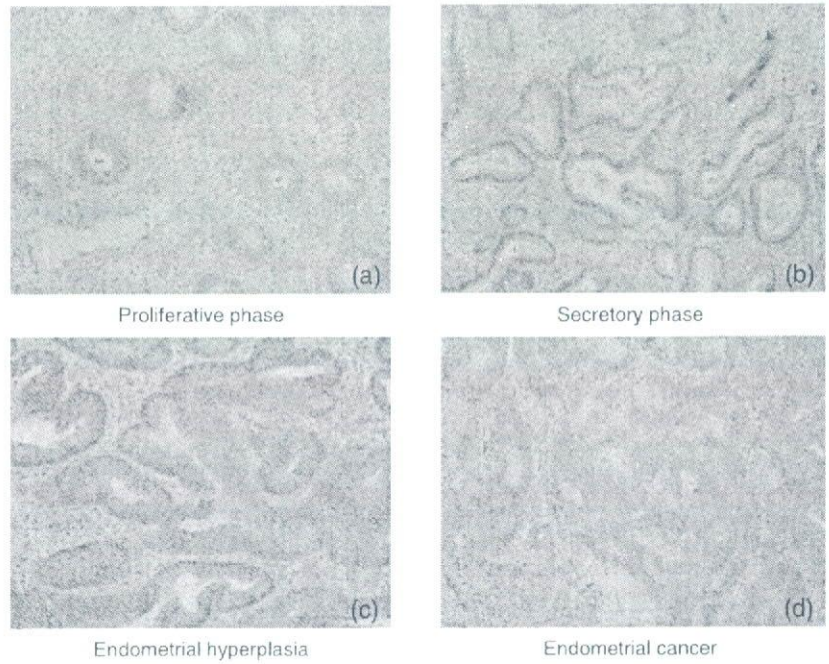

Fig. 3. Retinoic acid receptor (RAR) β localization in eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. (a,b) In ectopic endometrium, RARβ immunoreactivities were detected in the nuclei of epithelial cells throughout all menstrual phases. (c) In endometrial hyperplasia, RARβ immunoreactivity was detected in the nuclei of 60–75% of stromal cells. In endometrial carcinoma, no RARβ immunoreactivity was detected in the stromal cells in any of the cases examined. (d) RARβ immunoreactivities were detected in the nuclei of 18–37% of carcinoma cells. Immunohistochemistry for the RAR was carried out using serial sections of eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. (Original magnification, ×200.)