Omoto Y, Kobayashi Y, Nishida K, Tsuchiya E, Eguchi H, Nakagawa K, Ishikawa Y, Yamori T, Iwase H, Fujii Y *et al.* 2001 Expression, function, and clinical implications of the estrogen receptor beta in human lung cancers. *Biochemical and Biophysical Research Communications* **285** 340–347.

O'Toole SA, Dunn E, Shppard BL, Sheils O, O'Leary JJ, Wuttke W & Seidlova-Wuttke D 2005 Oestrogen regulated gene expression in normal and malignant endometrial tissue. *Maturitas* **51** 187–198.

Pasqualini JR & Chetrite GS 2006 Estradiol as an anti-aromatase agent in human breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **98** 12–17.

Rose PG, Brunetto VL, Vanle L, Bell J, Walker JL & Lee RB 2000 A phase II trial of anastrozole in advanced recurrent or persistent endometrial carcinoma: a gynecologic oncology group study. *Gynecologic Oncology* **78** 212–216.

Rowan BG, Weigel NL & O'Malley BW 2000 Phosphorylation of steroid receptor coactivator-1: identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. *Journal of Biological Chemistry* **275** 4475–4483.

Rutanen EM, Pekonen F & Wahlstrom T 1993 Insulin-like growth factors and their binding proteins in benign and malignant uterine diseases. *Growth Regulation* **3** 74–77.

Sasano H, Kaga K, Sato S, Yajima A, Naura H & Harada N 1996 Aromatase cytochrome P450 gene expression in endometrial carcinoma. *British Journal of Cancer* **74** 1541–1544.

Segawa T, Shozu M, Murakami K, Kasai T, Shinohara K, Nomura K, Ohno S & Inoue M 2005 Aromatase expression in stromal cells of endometrioid endometrial cancer correlates with poor survival. *Clinical Cancer Research* **11** 2188–2194.

Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC, Winder AD, Reiter R, Wellstein A, Martin MB *et al.* 2003 Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI3-K/Akt pathway. *Oncogene* **22** 7998–8011.

Takase Y, Levesque MH, Luu-The V, El-Alfy M, Labrie F & Pelletier G 2006 Expression of enzymes involved in estrogen metabolism in human prostate. *Journal of Histochemistry and Cytochemistry* **54** 911–921.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034.

Watanabe K, Sasano H, Harada N, Ozaki M, Niikura H, Sato S & Yajima A 1995 Aromatase in human endometrial carcinoma and hyperplasia. Immunohistochemical, *in situ* hybridization, and biochemical studies. *American Journal of Pathology* **146** 491–500.

Yamaguchi Y, Takei H, Suemasu K, Kobayashi Y, Kurosumi M, Harada N & Hayashi S 2005 Tumor–stromal interaction through the estrogen-signaling pathway in human breast cancer. *Cancer Research* **65** 1653–1662.

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Intratumoral Estrogens and Estrogen Receptors in Human Non–Small Cell Lung Carcinoma

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Abstract **Purpose:** The possible involvement of gender-dependent factors has been suggested in human non-small cell lung carcinomas (NSCLC), but their precise roles remain largely unclear. Therefore, we examined intratumoral estradiol concentrations in NSCLC to examine local actions of estrogens in NSCLC.

Experimental Design: Fifty-nine frozen specimens of NSCLC were available for liquid chromatography/electrospray tandem mass spectrometry to study intratumoral estradiol concentrations. In addition, A549 NSCLC cells stably expressing estrogen receptor (ER) α (A549 + ERα) or ERβ (A549 + ERβ) were used *in vitro* studies.

Results: Forty-three (73%) of 59 NSCLC showed higher concentration of estradiol in carcinoma tissues than the corresponding nonneoplastic lung tissues from the same patient, and intratumoral estradiol concentrations were significantly ($P = 0.0002$ and 2.2-fold) higher than the corresponding nonneoplastic lungs. The intratumoral concentration of estradiol was positively correlated with aromatase expression, tumor size, and Ki-67 status in ERα- or ERβ-positive cases. In *in vitro* studies, estradiol significantly increased cell proliferation of A549 + ERα or A549 + ERβ, which was significantly suppressed by selective ER modulators, tamoxifen or raloxifene. Both A549 + ERα and A549 + ERβ cells expressed aromatase. The cell proliferation level in these cells was significantly increased under treatment with testosterone, and it was inhibited by addition of the aromatase inhibitor letrozole.

Conclusions: These results suggest that estradiol is locally produced in NSCLC mainly by aromatase and plays an important role in the growth of ERα- or ERβ-positive NSCLC. Therefore, use of selective ER modulators and/or aromatase inhibitors may be clinically effective in NSCLC that are positive for both ER and aromatase.

Lung carcinoma is the most leading cause of cancer mortality throughout the world. Despite recent improvements in its treatment, it still remains a highly lethal disease (1–3). Therefore, it is very important to investigate biological features of the lung cancer to develop targeted therapies aimed at specific proteins expressed by the carcinoma cells. Lung carcinoma is histologically classified into small cell carcinoma and non-small cell lung carcinoma (NSCLC). NSCLC accounts for ~80% of the lung carcinomas and is composed of heterogeneous groups such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Squamous cell carcinoma is closely associated with smoking and a higher frequency is detected in men, whereas adenocarcinoma tends to occur more frequently in women, suggesting a possible involvement of gender-dependent factors in the pathogenesis of NSCLC (1, 4, 5).

It is well known that sex steroids play important roles in various human tissues as gender-dependent factors including nonclassic target tissues. Among sex steroids, estrogens are major contributors to cell proliferation of both breast and endometrial carcinomas through an initial interaction with estrogen receptor (ER) α and/or ERβ. Previous studies showed that a great majority of NSCLC expressed ERs (5–8) and estrogen stimulated the growth of NSCLC tumors in nude mouse xenografts. In addition, hormone replacement therapy has been reported to significantly decrease survival in women with lung cancer (9). Therefore, estrogenic actions have been postulated to contribute to the development and/or progression of NSCLC.

The most biologically active estrogen is estradiol; therefore, it is very important to examine the intratumoral concentrations of estradiol in NSCLC to obtain a better understanding of estrogenic actions in NSCLC. However, measurements of intratumoral estrogen concentrations have not been reported

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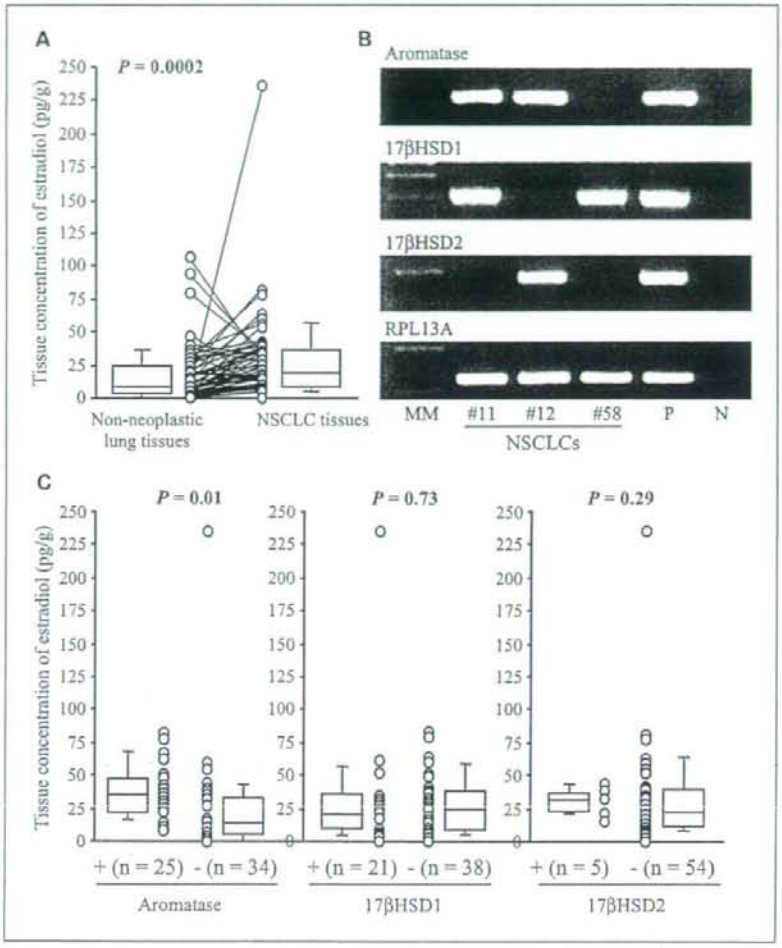

Fig. 1. Intratumoral concentration of estradiol in 59 NSCLC. *A*, tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues. Each value was shown in an open circle, and the paired values from the same patient were connected in a line. The grouped data are represented as box-and-whisker plots. The median value is shown by a horizontal line in the box plot and the gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The upper and lower bars indicate the 90th and 10th percentiles, respectively. The statistical analysis was done by a Wilcoxon matched-pair signed-rank test. *B*, RT-PCR for enzymes related to estrogen production in three representative NSCLC tissues. mRNA expression of RPL13A was also evaluated as an internal standard. *MM*, molecular marker; *P*, positive control (placental tissues); *N*, negative control (no cDNA substrate). *C*, association between intratumoral concentration of estradiol and enzymes related to estrogen production in the NSCLC. Values of each case were represented as an open circle and the grouped data were shown as box-and-whisker plots. Expression of aromatase, 17βHSD1, and 17βHSD2 was evaluated by RT-PCR analyses. The statistical analysis was done by a Mann-Whitney's *U* test. *P* values < 0.05 were considered significant and were indicated in boldface.

at all in NSCLC, so the clinical and/or biological significance of the role of estrogens have largely remained unknown in NSCLC. Therefore, in this study, we first measured the tissue concentration of estradiol in 59 cases of NSCLC and correlated these findings with various clinicopathologic factors of the cases. We subsequently characterized the potential biological functions of estrogens in NSCLC cells through the use of cell culture studies.

Materials and Methods

Patients and tissue specimens. Fifty-nine specimens of NSCLC were obtained from patients who underwent surgical resection from 2000 to 2003 in the Department of Surgery at Tohoku University Hospital. Thirty-three patients were men and the mean age was 71 years (range, 45-82 years), whereas 26 cases were from postmenopausal women and the mean age was 71 years (range, 50-81 years). NSCLC tissue from premenopausal women was not available for examination in this study. The patients examined in this study did not receive irradiation or chemotherapy before surgery. Overall survival data were available for all patients examined, with the mean follow-up time of 1,257 days [3.4 years; range, 245-2,414 days (0.7-6.6 years)]. Specimens for estradiol extraction or RNA isolation were snap-frozen and stored at -80°C and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax.

Informed consent was obtained from all the patients before their surgery and examination of the specimens used in this study. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine.

Liquid chromatography/electrospray tandem mass spectrometry. Concentrations of estradiol were measured by liquid chromatography/electrospray tandem mass spectrometry analysis in Teizo Medical as described previously (10, 11). Briefly, we used a liquid chromatograph (Agilent 1100, Agilent Technologies) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems) operated with electrospray ionization in the positive-ion mode in this study. The mobile phase consisted of solvents A [0.1% formic acid in water (v/v)] and B (acetonitrile) and delivered at flow rate of 0.4 mL/min. We used mixture of solvents A and B [30:70 (v/v)] as an initial condition. After injection, it was followed by a linear

gradient to 100% solvent B for 4 min, and this condition was maintained for 3 min. The system was returned to the initial proportion within 0.05 min and maintained for the final 2.95 min of each run. For multiple reaction monitoring mode, the instrument monitored the m/z 255.3 (I.S. 258.3) from 396.4 (I.S. 399.4) for estradiol derivatives.

In our present study, the lower limit of quantification of estradiol was 0.2 pg. The reproducibility of the experiment was evaluated by intra-assay and interassay ($n = 3$), and their coefficient variations were 12% and 2%, respectively.

Reverse transcription-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) and a reverse transcription kit (SuperScript II, Preamplification System, Life Technologies) was used in the synthesis of cDNA. Reverse transcription-PCR (RT-PCR) was done using the Light-Cycler System (Roche Diagnostics) and ribosomal protein L 13a (RPL13A) was also used as an internal standard. The primer sequences used in this study are as follows: aromatase (X13589; forward: cDNA position 691-711 and reverse: cDNA position 766-786; ref. 11), 17β-hydroxysteroid dehydrogenase (17βHSD) type 1 (17βHSD1) (NM_000413; forward: 1,290-1,310 and reverse: 1,604-1,623), 17βHSD type 2 (17βHSD1; NM_002153; forward: 797-816 and reverse: 971-989), ERα (NM_000125; forward: 1,811-1,831 and reverse: 2,080-2,100), ERβ (AB006590; forward: 1,460-1,480 and reverse: 1,608-1,627), and RPL13A (NM_012423; forward: 487-509 and reverse: 588-612; ref. 12). To verify amplification of the correct sequences, the PCR products were purified and subjected to direct sequencing. Negative controls, in which the reaction mixture lacked cDNA template, were included to check for the possibility of exogenous contaminant DNA.

Immunohistochemistry. Monoclonal antibodies for ERα (NCLER-6F11), ERβ (MS-ERβ13-PX1), and Ki-67 (MIB1) were purchased from Novocastra Lab, Gene Tex, and DAKO, respectively. Rabbit polyclonal antibody for 17βHSD1 was kindly provided by Dr. M. Poutanen (University of Oulu; ref. 13). In this study, a Histofine Kit (Nichirei), which employs the streptavidin-biotin amplification method was used for immunohistochemistry. Antigen retrieval was done by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer [2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate (pH 6.0)] for ERα, ERβ, and Ki-67 immunostaining. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mmol/L 3,3′-diaminobenzidine, 50 mmol/L Tris-HCl (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibody.

Immunoreactivity for ERα, ERβ, and Ki-67 was detected in nuclei of carcinoma cells and the immunoreactivity was evaluated in >1,000 carcinoma cells for each case. Subsequently, the percentage of immunoreactivity, labeling index (LI), was determined. Cases with ERα or ERβ LI of >10% were considered ERα- or ERβ-positive NSCLC as reported previously (14). Immunoreactivity for 17βHSD1 was detected in the cytoplasm of carcinoma cells, and cases that had >10% of positive carcinoma cells were considered positive (15).

To evaluate relative immunointensity of ERs in NSCLC, immunoreactivity of ERα and ERβ was also evaluated by H scoring system (16). Briefly, ER-positive carcinoma cells were further classified into the strongly or weakly positive cells, and H scores were subsequently generated by adding together 2× % strongly stained cells, 1× % weakly stained cells, and 0× % negative cells, giving a possible range of 0 to 200.

Cell culture and chemicals. Human NSCLC cell line A549 was purchased from Institute of Development, Aging and Cancer, Tohoku University. The A549 cells were cultured in RPMI 1640 (Sigma-Aldrich)

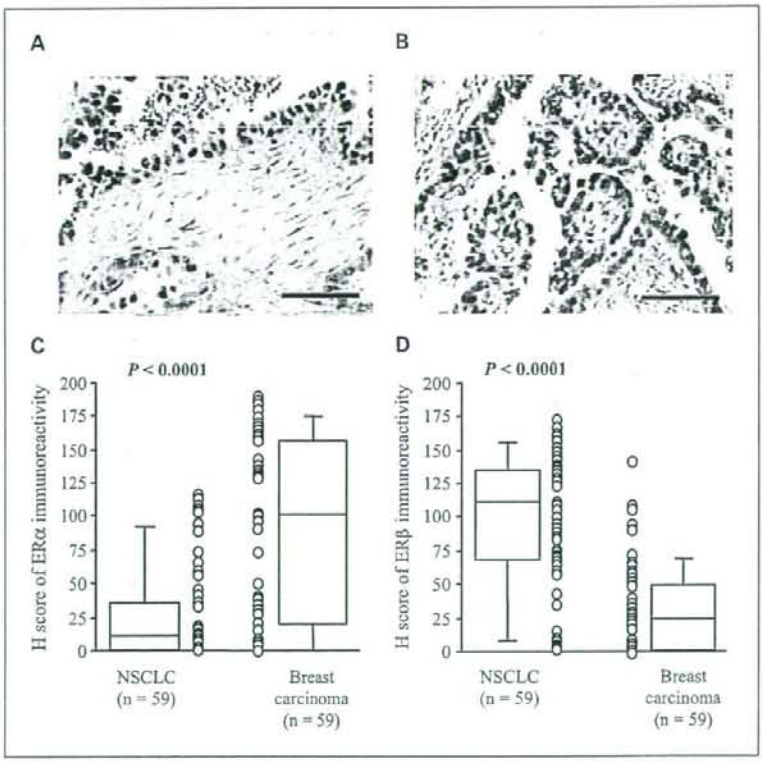

Fig. 2. Immunohistochemistry for ER isoforms in 59 NSCLC. *A* and *B*, immunoreactivity of ERα (*A*) and ERβ (*B*) was detected in the nucleus of adenocarcinoma cells in NSCLC. Bar, 50 μm. *C* and *D*, immunointensity of ERα (*C*) or ERβ (*D*) in NSCLC compared with breast carcinomas. Each value was represented as an open circle and the grouped data were shown as box-and-whisker plots. The statistical analysis was done by a Mann-Whitney's *U* test.

Table 1. Association between intratumoral concentration of estradiol and clinicopathologic variables according to ERα status in 59 NSCLC

Value	Intratumoral concentration of estradiol					
	ERα-positive cases (n = 32)			ERα-negative cases (n = 27)		
	n	Median (minimum-maximum)	P	n	Median (minimum-maximum)	P
Patient age* [70 (45-82) y]	32	22 (0-234)	0.23	27	18 (0-78)	0.55
Gender						
Men	15	32 (13-234)		18	35 (9-78)	
Postmenopausal women	17	10 (0-50)	**0.0002**	9	6 (0-17)	**0.001**
Stage						
I	19	18 (0-234)		15	15 (0-60)	
II-IV	13	30 (0-65)	0.24	12	33 (4-78)	0.43
Tumor size* [2.8 (1.0-6.5) cm]	32	22 (0-234)	**0.01** (r = 0.45)	27	18 (0-78)	0.70
Histologic type						
Adenocarcinoma	28	20 (0-234)		16	15 (0-78)	
Squamous cell carcinoma	4	31 (6-32)	0.78	11	31 (9-77)	0.20
Lymph node metastasis						
Positive	9	30 (0-65)		6	15 (5-77)	
Negative	23	18 (0-234)	0.41	21	22 (0-78)	0.73
Ki-67 LI* [18% (0-54%)]	32	22 (0-234)	**0.01** (r = 0.47)	27	18 (0-78)	0.19

Data were statistically analyzed using Mann-Whitney's U test or the Spearman rank correction. P values < 0.05 were considered significant and are indicated in boldface. Indicate which statistical methods were used for which particular data.
*Data were continuous variable and the median with minimum-maximum values was presented.

with 10% fetal bovine serum (JRH Biosciences). In this study, cells were cultured with phenol red–free RPMI 1640 containing 10% dextran-coated charcoal-fetal bovine serum for 3 days before the experiment.

Sex steroids (estradiol and testosterone) and selective ER modulators (SERM) such as tamoxifen and raloxifene were purchased from Sigma-Aldrich. An ERα agonist (propyl-pyrazole-triol; PPT; ref. 17), ERβ agonist (diarylpropionitrile; DPN; ref. 17), and pure ER antagonist (ICI 182,780) were purchased from Tocris. The aromatase inhibitor letrozole was synthesized within laboratories at Novartis Pharma.

Stable transfection. Stable transfection was done according to previous reports with some modifications (5, 18), and ERα or ERβ expression vector for ERα (pRc/CMV-ERα) or ERβ (pRc/CMV-ERβ)

Table 2. Association between intratumoral concentration of estradiol and clinicopathologic variables according to ERβ status in 59 NSCLC

Value	Intratumoral concentration of estradiol					
	ERβ-positive cases (n = 53)			ERβ-negative cases (n = 6)		
	n	Median (minimum-maximum)	P	n	Median (minimum-maximum)	P
Patient age [70 (45-82) y]	53	20 (0-234)	0.17	6	34 (15-40)	0.80
Gender						
Men	28	34 (9-234)		5	36 (18-40)	
Postmenopausal women	25	9 (0-50)	**<0.0001**	1	15	NA
Stage						
I	30	15 (0-234)		4	37 (15-40)	
II-IV	23	32 (0-78)	0.09	2	24 (18-31)	0.35
Tumor size [2.8 (1.0-6.5) cm]	53	20 (0-234)	**0.04** (r = 0.27)	6	34 (15-40)	0.34
Histologic type						
Adenocarcinoma	42	190 (0-234)		2	26 (15-37)	
Squamous cell carcinoma	11	30 (6-77)	0.51	4	34 (18-40)	0.64
Lymph node metastasis						
Positive	14	30 (0-77)		1	18	
Negative	39	18 (0-234)	0.53	5	36 (15-40)	NA
Ki-67 LI [18% (0-54%)]	53	20 (0-234)	**0.01** (r = 0.37)	6	34 (15-40)	0.78

NOTE: Data were continuous variable, and the median with minimum-maximum values were presented.
NA indicates that the P value was not available because of n = 1 in one group. Data were statistically analyzed using Mann-Whitney's U test or the Spearman rank correction. P values < 0.05 were considered significant and are indicated in boldface.

used in this study was described previously (5, 18). Briefly, A549 cells were transfected with ERα or ERβ expression vector with Trans IT LT-1 reagent (Takara), respectively. After 24 h in culture, the cells were grown in fresh RPMI 1640 supplemented with 10% fetal bovine serum containing 1 mg/mL geneticin (G418; Sigma-Aldrich) for 2 weeks. Isolated colonies were trypsinized in metal ring cups and the cells were further cultured in the presence of 200 μg/mL G418. As a negative control, empty vector was also transfected in the A549 cells.

Luciferase assay. The luciferase reporter gene assay was done as described previously (19) with some modifications. Briefly, 1 μg ptk-ERE-Luc plasmids and 200 ng pRL-TK control plasmids (Promega) were used to measure the transcriptional activity of ER. Transient transfections were carried out using *Trans*IT-LT Transfection Reagents (TaKaRa) in A549 transformants and the luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200; ATTO). The transfection efficiency was normalized against *Renilla* luciferase activity using pRL-TK control plasmids and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls.

Cell proliferation assays. A549 transformants were treated with the indicated compounds for 3 days and the status of cell proliferation was measured by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] method using Cell Counting Kit-8 (Dosin Kagaku; ref. 12).

Results

Intratumoral estradiol concentrations in NSCLC. We first examined the tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues using liquid chromatography/electrospray tandem mass spectrometry. As shown in Fig. 1A, the median with minimum-maximum value of tissue concentration of estradiol was 20 (0-234) pg/g in NSCLC. Forty-three (73%) of 59 NSCLC cases showed higher concentration of estradiol in carcinoma tissues than the corresponding nonneoplastic lung tissues from the same patients, and the intratumoral estradiol concentrations were significantly ($P = 0.0002$ and 2.2-fold) higher than those found in their corresponding nonneoplastic lung tissues [9 (0-116) pg/g]. This correlation was detected regardless of the gender of the patients

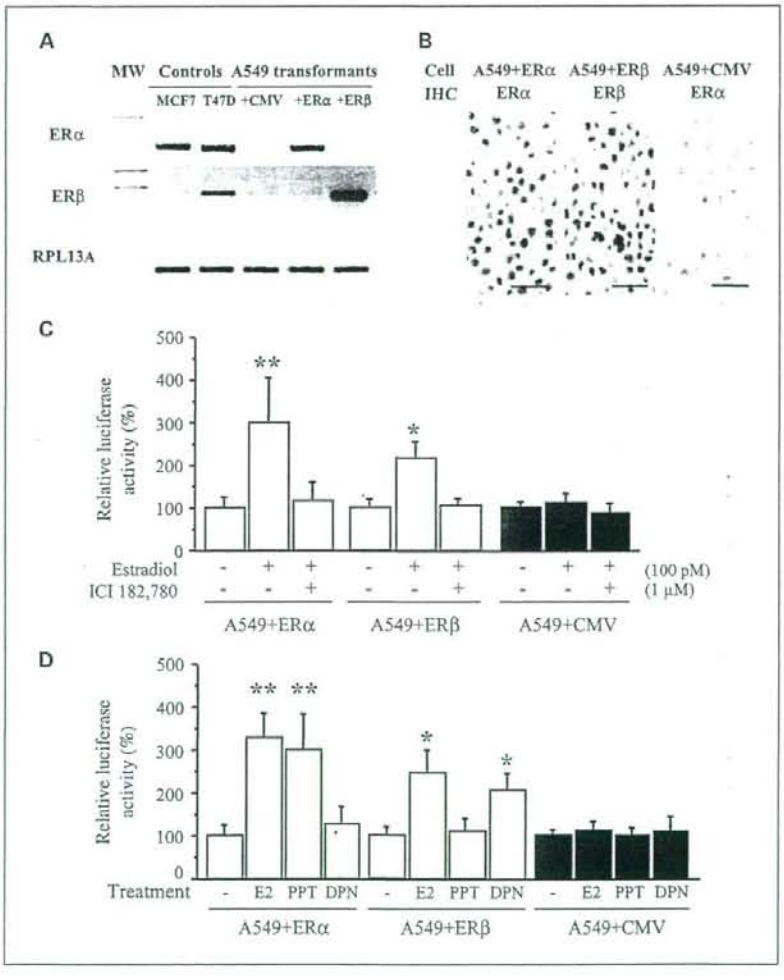

Fig. 3. Expression of ER isoforms in transformant A549 human NSCLC cells. *A*, A549 + ERα cells expressed ERα mRNA, whereas A549 + ERβ cells expressed ERβ mRNA. A549 + CMV cells did not express either ERα or ERβ mRNA. mRNA expression of ER isoforms was examined by RT-PCR analysis. As positive controls, MCF-7 breast carcinoma cells for ERα and T-47D breast carcinomas for ERα and ERβ were used. As an internal standard in each sample, RT-PCR was done for RPL13A. *B*, immunohistochemistry for ER isoforms in the A549 transformants. Immunoreactivity of ERα was detected in the nucleus of A549 + ERα cells (*left*), whereas that of ERβ was positive in the nucleus of A549 + ERβ cells (*middle*). No significant immunoreactivity for ER isoforms was detected in A549 + CMV cells (*right*). Immunoreactivity was done using cell blocks from formalin-fixed and paraffin-embedded specimens. Bar, 25 μm. *C* and *D*, estrogen-responsive element – dependent transactivation by estradiol in A549 cells stably expressing ER isoforms. A549 transformants were transiently transfected with ptk-ERE-Luc plasmids and treated with estradiol (100 pmol/L) and/or an ER antagonist ICI 182,780 (1 μmol/L; *C*), or estradiol (100 pmol/L), ERα agonist PPT (100 pmol/L), or ERβ agonist DPN (100 pmol/L; *D*) for 24 h. Subsequently, the luciferase assay was done. The luciferase activity was evaluated as a ratio (%) compared with that of controls (no treatment with either estradiol or ICI 182,780 for 24 h). Mean ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ versus controls, respectively (one-way ANOVA and Fisher's protected least significant difference test).

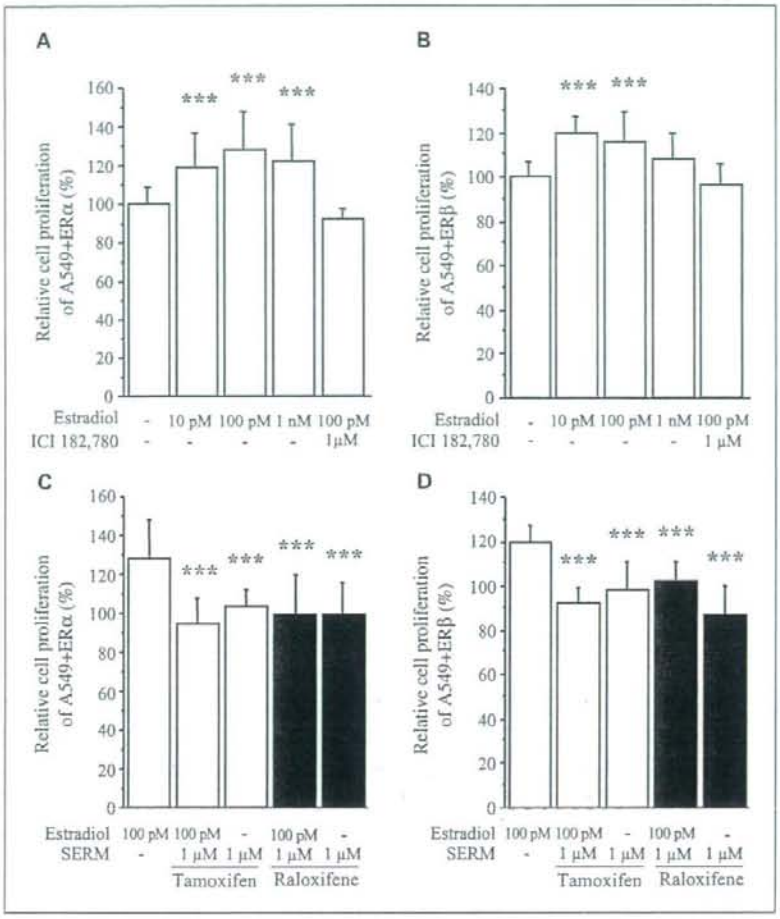

Fig. 4. Effects of ER isoform expression on the estrogen-mediated proliferation of A549 transformants. A549 + ERα (*A*) and A549 + ERβ (*B*) cells were treated with the indicated concentrations of estradiol and/or ICI 182,780 (1 μmol/L) in phenol red–free RPMI 1640 containing 10% dextran-coated charcoal-fetal bovine serum for 3 d. The status of cell proliferation was measured using a WST-8 method. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either estradiol or ICI 182,780). Mean ± SD (n = 6). ***, $P < 0.001$ versus controls (one-way ANOVA and Fisher's protected least significant difference test). To examine the effects of SERMs on the estradiol-mediated proliferation of A549 cells expressing ER isoforms, A549 + ERα (*C*) and A549 + ERβ (*D*) cells were treated with estradiol (100 pmol/L) and/or SERM [tamoxifen (1 μmol/L) or raloxifene (1 μmol/L)] for 3 d. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either estradiol or SERM). Mean ± SD (n = 6). ***, $P <$ 0.001 versus estradiol (100 pmol/L) alone (one-way ANOVA and Fisher's protected least significant difference test).

(P = 0.004 and 1.7-fold in men and P = 0.01 and 2.3-fold in postmenopausal women). Tissue concentrations of estradiol in men were significantly higher than that found in postmenopausal women both in NSCLC ($P < 0.0001$ and 3.9-fold [35 (9-234) pg/g in men and 9 (0-50) pg/g in postmenopausal women]) and nonneoplastic lung tissues ($P < 0.0001$ and 5.3-fold [21 (0-116) pg/g in men and 4 (0-32) pg/g in postmenopausal women]).

We then examined the association between the intratumoral estradiol concentration and expression of enzymes related to estrogen production in NSCLC tissues. mRNA expression for aromatase, 17βHSD1, 17βHSD2, and RPL13A was detected as a specific single band (115, 326, 192, and 125 bp, respectively) by RT-PCR analyses (Fig. 1B), and the results for 17βHSD1 were confirmed by immunohistochemistry (data not shown). As shown in Fig. 1C, the intratumoral estradiol concentration was significantly associated with aromatase (P = 0.01) but not with 17βHSD1 (P = 0.73) or 17βHSD2 (P = 0.29).

Association between intratumoral concentration of estradiol and clinicopathologic variables according to ER status in NSCLC. We subsequently examined an association between intratumoral estradiol concentration and clinicopathologic factors according to ER status in NSCLC, because estrogenic actions are mediated through an interaction with estradiol and ER isoforms. Immunoreactivity for both ERα (Fig. 2A) and ERβ (Fig. 2B) was detected in 32 (54%) and 53 (90%) of the 59 NSCLC cases, respectively, and 54 (92%) of 59 cases were ER (ERα or ERβ) positive. The intratumoral concentration of estradiol was not significantly associated with the ERα (P = 0.96) or ERβ status (P = 0.28).

When we evaluated relative immunointensity of ERs by H score in NSCLC and correlated with that in the same number of breast carcinomas, the relative immunointensity of ERα was significantly ($P < 0.0001$ and 9.3-fold) lower in NSCLC than the breast carcinomas (Fig. 2C), whereas ERβ immunointensity in NSCLC was significantly ($P < 0.0001$ and 4.7-fold) higher (Fig. 2D).

As shown in Table 1, the intratumoral estradiol concentration was positively associated with tumor size (P = 0.01) and Ki-67 LI (P = 0.01) in ERα-positive NSCLC but not in

ERα-negative cases ($P = 0.70$ in tumor size and $P = 0.19$ in Ki-67 LI). The intratumoral concentration of estradiol was significantly higher in men than postmenopausal women regardless of ERα status ($P = 0.0002$ in ERα-positive group and $P = 0.001$ in ERα-negative group). No significant association was detected between intratumoral estradiol concentration and other clinicopathologic factors examined regardless of the ERα status of the carcinoma cells. Estradiol concentration in NSCLC was also positively associated with tumor size ($P = 0.04$) and Ki-67 LI ($P = 0.01$) in ERβ-positive NSCLC and was significantly higher in male patients than postmenopausal women ($P < 0.0001$; Table 2).

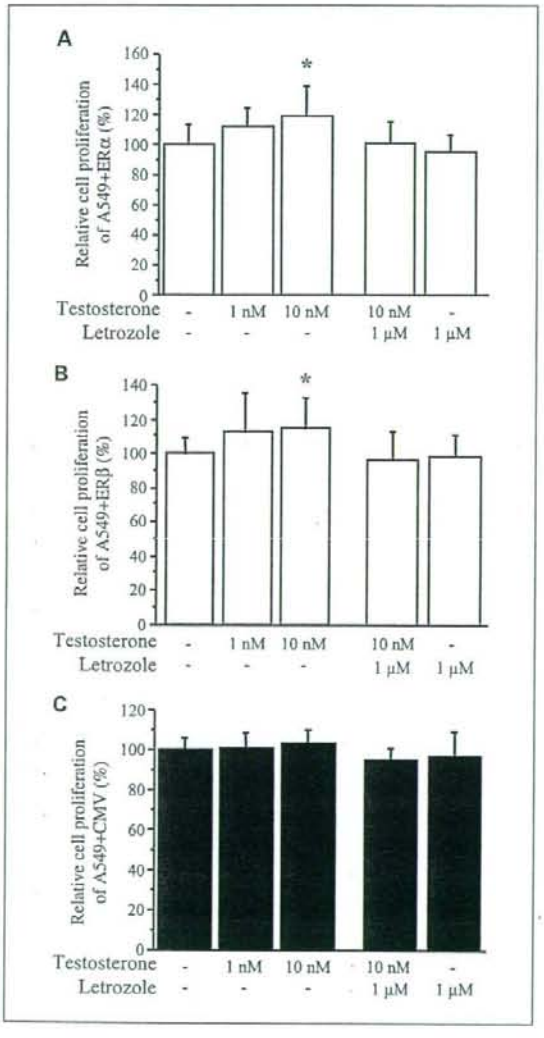

Fig. 5. Effects of the aromatase inhibitor letrozole on the cell proliferation of A549 cells expressing ER isoforms. A549 + ERα (*A*), A549 + ERβ (*B*), and A549 + CMV (*C*) cells were treated with indicated concentration of testosterone and/or letrozole for 3 d. The status of cell proliferation was measured using a WST-8 method. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either testosterone or letrozole). Mean ± SD ($n = 4$). *, $P < 0.001$ versus controls (one-way ANOVA and Fisher's protected least significant difference test).

When the intratumoral concentration of estradiol was categorized into two groups according to their median values, the higher concentration group tended to be associated with worse prognosis in ER-positive NSCLC cases, although the association did not reach significant level ($P = 0.12$, log-rank test) but not in ER-negative patients ($P = 0.59$) in this study.

Establishment of A549 NSCLC cells expressing ERα or ERβ. To further characterize the biological functions of ER isoforms in NSCLC, transformed A549 NSCLC cells expressing ERα (A549 + ERα) or ERβ (A549 + ERβ) were established (Fig. 3A), because the parental A549 cells examined in this study did not express ERα or ERβ at both mRNA and protein levels in our study (data not shown). As a control, we also isolated a clone named A549 + CMV, which was stably transfected with empty vector in the A549 cells. mRNA expression of ER isoforms was not detected in the A549 + CMV cells (Fig. 3A). The patterns of protein expression of ER isoforms in these cells were confirmed by immunohistochemistry (Fig. 3B).

We subsequently examined the effects of ERα or ERβ expression in these cells on the transcriptional activity mediated through estrogen-responsive element using luciferase reporter gene assays. When the A549 transformants were transiently transfected with ptk-ERE-Luc plasmids and treated with estradiol (100 pmol/L), the luciferase activity was significantly increased in A549 + ERα ($P < 0.01$ and 3.2-fold) and A549 + ERβ ($P < 0.05$ and 2.1-fold) cells but not in A549 + CMV cells (1.1-fold) when compared with their basal levels (no estradiol treatment; Fig. 3C). The estrogen-responsive element–dependent transactivation by estradiol in A549 + ERα or A549 + ERβ cells was significantly inhibited ($P < 0.01$ and $P < 0.05$, respectively) by addition of the ER antagonist ICI 182,780. The luciferase activity was significantly ($P < 0.01$ and 3.0-fold) increased by the treatment with ERα agonist PPT (100 pmol/L), but not ERβ agonist DPN (100 pmol/L), in A549 + ERα cells, whereas the activity was significantly ($P < 0.05$ and 2.0-fold) induced by DPN, but not PPT, in A549 + ERβ cells (Fig. 3D).

When parental A549 cells were treated with estradiol, PPT, or DPN (100 pmol/L, respectively), the estrogen-responsive element–dependent transactivation was not significantly increased (1.1-, 1.0-, or 1.1-fold) compared with the basal level in our present study.

Effects of ERα or ERβ expression on estrogen-mediated proliferation in A549 cells. The number of A549 + ERα cells was significantly increased following the treatment with estradiol over the concentration range of 10 pmol/L to 1 nmol/L for 3 days (Fig. 4A). The cell proliferation of A549 + ERα cells treated with 100 pmol/L estradiol was 1.3-fold higher than the basal proliferative level measured in the absence of estradiol ($P < 0.001$). Estradiol-induced cell proliferation was significantly inhibited ($P < 0.001$) by addition of ICI 182,780 (1 μmol/L), with proliferation comparable with the basal levels being observed.

Estradiol-mediated cell proliferation was also detected in A549 + ERβ cells and was significantly induced following treatment with 10 and 100 pmol/L estradiol ($P < 0.001$; Fig. 4B). The estradiol-mediated cell proliferation of A549 + ERβ cells was significantly inhibited ($P < 0.001$) by the addition of ICI 182,780 (1 μmol/L) with proliferation comparable with

the basal levels being observed. In the A549 + CMV and parental A549 cells, estradiol, PPT, or DPN (100 pmol/L, respectively) did not significantly change the basal cell proliferation (data not shown).

Effects of SERMs on estrogen-mediated proliferation in A549 cells expressing ER isoform. As shown in Fig. 4C, the estradiol-mediated proliferation of A549 + ERα cells was significantly suppressed by addition of SERMs such as tamoxifen (1 μmol/L) or raloxifene (1 μmol/L; $P < 0.001$). Tamoxifen or raloxifene alone did not significantly change the status of cell proliferation of estradiol-untreated A549 + ERα cells.

Similarly, the estradiol-mediated proliferation of A549 + ERβ cells was also significantly inhibited by tamoxifen ($P < 0.001$) or raloxifene ($P < 0.001$; Fig. 4B). Tamoxifen or raloxifene did not significantly alter the cell proliferation level of estradiol-untreated A549 + ERβ cells. The cell proliferation of A549 + CMV or parental A549 cells was not significantly influenced following treatment with estradiol (100 pmol/L) and/or SERM (1 μmol/L; data not shown).

Aromatase in A549 cells expressing ER isoform and its correlation with cell proliferation. Using liquid chromatography/electrospray tandem mass spectrometry analysis of NSCLC, the intratumoral estradiol concentration was positively associated with the status of aromatase expression (Fig. 1C). Therefore, intratumoral aromatase may play an important role in contributing to the endogenous estrogen-mediated cell proliferation in NSCLC. To further validate this hypothesis, we used the A549 transformants, because these cells all expressed aromatase mRNA (data not shown) as reported previously (20).

When A549 + ERα or A549 + ERβ cells were treated with 10 nmol/L testosterone as a substrate for estrogen production by aromatase for 3 days, the number of the cells was significantly increased compared with the basal level (no treatment with testosterone; $P < 0.05$; Fig. 5A and B). This increased cell proliferation was significantly inhibited by the addition of the aromatase inhibitor letrozole in both A549 + ERα ($P < 0.05$) and A549 + ERβ ($P < 0.01$) cells (these significance levels are not indicated in the figure). Letrozole alone did not significantly change the status of the cell proliferation of A549 + ERα or A549 + ERβ cells. The cell proliferation of the A549 + CMV (Fig. 5C) or parental A549 (data not shown) cells was not significantly influenced by addition of testosterone and/or letrozole.

Discussion

To the best of our knowledge, this is the first report to have shown intratumoral concentrations of estradiol in NSCLC. In our present study, the median value of estradiol concentrations in NSCLC was 20 pg/g and was significantly higher (2.2-fold) than that found in the nonneoplastic lung tissues. Previously reported studies showed that estradiol is significantly (2.3-fold) higher in breast carcinomas in postmenopausal women (388 pg/g) than in the areas considered as morphologically normal (172 pg/g) in the same patients (21). Estradiol is considered to be locally produced from circulating inactive steroids found within the breast carcinoma tissues (22). The intratumoral concentration of estradiol in NSCLC was ~20 times lower than that detected in the breast carcinomas of postmenopausal women (21). However, the relative ratio of the intratumoral estradiol concentration to the corresponding nonneoplastic tissue of the same patients was similar between these two carcinomas (2.2 in NSCLC and 2.3 in breast carcinoma). Therefore, it is suggested that estradiol is also locally synthesized in NSCLC as in the great majority of breast carcinomas.

Results of our present study also showed that estradiol concentration in NSCLC tissues was significantly higher (3.7-fold) in men than postmenopausal women. Plasma concentrations of testosterone and androstendione are higher (~10 and 3 times, respectively) in men than postmenopausal women, whereas the plasma concentration of estrogens are similar (Endocrinology Databases; http://www.il-st-acad-sci.org/data2.html). Therefore, circulating androgens are considered to be the major precursor substrates of local estradiol production in NSCLC. In breast carcinoma tissues, estradiol is known to be locally produced from circulating inactive steroids by several sex steroid-producing enzymes including aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol; ref. 22). In our present study, the intratumoral estradiol concentration in NSCLC was positively associated with aromatase expression but not with the other examined enzymes that can potentially contribute to the production of intratumoral estrogens. Weinberg et al. (20) recently reported that aromatase was expressed in NSCLC cells at both mRNA and protein levels. Provost et al. (23) detected high level of 17βHSD activity in A549 cells and reported that 17βHSD5 was the predominant enzyme of the measured 17βHSD activity that is mainly involved in the regulation of intracellular androgen levels. Results of these studies as well as those in our present study indicate that the great majority of intratumoral estradiol is locally produced from circulating androgens by aromatase in NSCLC.

ER consists of ERα and ERβ in humans (3, 24, 25) and ERα is considered to mainly mediate estrogenic actions (26). A great majority of breast carcinomas are positive for ERα and SERMs such as tamoxifen or aromatase inhibitors such as letrozole are being used clinically as antiendocrine therapies for ERα-positive breast carcinoma patients. In NSCLC, ERβ immunoreactivity has been found to be frequently positive (6, 14, 27, 28), whereas the status of ERα immunoreactivity showed marked variability in its frequency of immunopositivity (0-73%) among the different studies reported (5, 14, 27, 29-31). In these previously reported investigations, the same ERα antibody employed in our present study (clone 6F11) was used in three groups, in which ERα positivity was 0% (30), 38% (14), and 66% (29). In addition, Dabbs et al. (29) reported that nuclear ERα immunoreactivity was detected with the 6F11 clone but not with the 1D5 clone, and these authors suggested that variability in ERα immunoreactivity might be due to different epitope recognized by the antibodies used in the study. In our study, ERα immunoreactivity was detected in 54% of NSCLC, but the relative immunointensity of ERα was much (9.3-fold and $P < 0.0001$) lower than that in the breast carcinoma examined. Thus, these results suggest that immunointensity of nuclear ERα is weak in NSCLC, which may result in variable interpretation of nuclear positivity and subsequently in inconsistent findings among the reports.

In previous *in vitro* studies, estrogens induced the proliferation of ER-expressing NSCLC cells, such as DB354, H23, and 201T cells (5, 6, 32). Stabile et al. (6) reported that estrogens stimulated tumor growth of H23 cells when injected into severe

combined immunodeficient mice. However, these cells all expressed both ERα and ERβ, so the biological significance of the different ER isoforms has still remained unclear in patients with NSCLC. In our present study, estradiol significantly increased the cell proliferation of A549 cells in the presence of ERα or ERβ. In addition, the intratumoral concentration of estradiol was significantly associated with tumor size and Ki-67 LI in both ERα- and ERβ-positive NSCLC but not in ER-negative cases. The MIB1 antibody for Ki-67 recognizes cells in all phases of the cell cycle except the G_0 (resting) phase (33), and Ki-67 LI is known to reflect the proliferative activity. Therefore, estrogens are reasonably postulated to contribute to the cell proliferation or other estrogen-dependent biological processes of NSCLC being mediated through both ERα or ERβ, which primarily occur in NSCLC tissues that are positive for both aromatase and ER.

If intratumoral estrogens promote the growth of NSCLC, antiestrogenic therapies would be considered to be effective in a selective group of NSCLC patients as in the breast carcinoma patients. In our present study, 1 μmol/L SERMs such as tamoxifen and raloxifene significantly suppressed the estradiol-mediated cell proliferation in both A549 + ERα or A549 + ERβ cells back to basal levels. Optimal concentrations of tamoxifen were generally considered to be 10 nmol/L to 10 μmol/L for *in vitro* studies (34) and serum concentrations of tamoxifen were reported to be 1.8 μmol/L in patients who received high-dose tamoxifen therapy (320 mg), although 20 mg tamoxifen is usually administrated in the great majority of breast carcinoma patients. In addition, the aromatase inhibitor letrozole also decreased the cell proliferation back to basal level in both A549 + ERα and A549 + ERβ cells treated with testosterone (Fig. 5). Weinberg et al. (20) showed that administration of the aromatase inhibitor anastrozole significantly reduced tumor growth of A549 cells in ovariectomized nude mouse xenografts, and very recently, Mah et al. (35) reported an association between aromatase expression and worse prognosis in women with early-stage NSCLC. Results of our present study using letrozole were in good agreements with those of the studies above. Therefore, tamoxifen and/or aromatase inhibitors would be considered to be clinically effective in ER-positive and aromatase-expressing NSCLC. The value of using antiendocrine therapies in NSCLC patients requires further examination.

In summary, the intratumoral estradiol concentration was significantly higher in NSCLC than nonneoplastic lung tissues of 59 examined cases. The estradiol concentration in NSCLC was associated with intratumoral aromatase and was correlated with both tumor size and Ki-67 in ERα- or ERβ-positive cases. In *in vitro* cell studies, estradiol significantly increased the cell proliferation of A549 cells stably expressing ER isoforms and this could be suppressed by addition of SERMs. The proliferation of these cells was also increased in the presence of testosterone and this was inhibited by the aromatase inhibitor, letrozole. Results from our present study suggest that estradiol is locally produced in NSCLC mainly through intratumoral aromatase and plays an important role in the growth of ER-positive NSCLC. Therefore, SERMs and/or aromatase inhibitors may be clinically effective in NSCLC patients who are positive for both ER and aromatase.

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References

1. Sekine I, Nishiwaki Y, Yokose T, Nagai K, Suzuki K, Kodama T. Young lung cancer patients in Japan: different characteristics between the sexes. Ann Thorac Surg 1999;67:1451–5.
2. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
3. Jemal A, Chu KC, Tarone RE. Recent trends in lung cancer mortality in the United States. J Natl Cancer Inst 2001;93:277–83.
4. Rom WN, Hay JG, Lee TC, Jiang Y, Tchou-Wong KM. Molecular and genetic aspects of lung cancer. Am J Respir Crit Care Med 2000;161:1355–67.
5. Omoto Y, Kobayashi Y, Nishida K, et al. Expression, function, and clinical implications of the estrogen receptor β in human lung cancers. Biochem Biophys Res Commun 2001;285:340–7.
6. Stabile LP, Davis AL, Gubish CT, et al. Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor α and β and show biological responses to estrogen. Cancer Res 2002;62:2141–50.
7. Mollerup S, Jorgensen K, Berge G, Haugen A. Expression of estrogen receptors α and β in human lung tissue and cell lines. Lung Cancer 2002;37:153–9.
8. Pietras RJ, Márquez DC, Chen HW, Tsai E, Weinberg O, Fishbein M. Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. Steroids 2005;70:372–81. Epub 2005 Mar 25.
9. Ganti AK, Sahmoun AE, Panwalkar AW, Tendulkar KK, Potti A. Hormone replacement therapy is associated with decreased survival in women with lung cancer. J Clin Oncol 2006;24:59–63. Epub 2005 Nov 28.
10. Suzuki T, Miki Y, Moriya T, et al. 5α-Reductase type 1 and aromatase in breast carcinoma as regulators of *in situ* androgen production. Int J Cancer 2007;120:285–91.
11. Miki Y, Suzuki T, Tazawa C, et al. Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. Cancer Res 2007;67:3945–54.
12. Suzuki T, Inoue A, Miki Y, et al. Early growth responsive gene 3 (EGR3) in human breast carcinoma: a regulator of estrogen-meditated invasion and a potent prognostic factor. Endocr Relat Cancer 2007;14:279–92.
13. Suzuki T, Moriya T, Ariga N, Kaneko C, Kanazawa M, Sasano H. 17β-Hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. Br J Cancer 2000;82:518–23.
14. Ishibashi H, Suzuki T, Suzuki S, et al. Progesterone receptor in non-small cell lung cancer—a potent prognostic factor and possible target for endocrine therapy. Cancer Res 2005;65:6450–8.
15. Suzuki T, Miki Y, Moriya T, et al. 5α-Reductase type1 and aromatase in breast carcinoma as regulators of *in situ* androgen production. Int J Cancer 2007;120:285–91.
16. Suzuki T, Takahashi K, Darnel AD, et al. COUP-TFII in the human adrenal cortex and its disorders. J Clin Endocrinol Metab 2000;85:2752–7.
17. Gottfried-Blackmore A, Croft G, McEwen BS, Bulloch K. Transcriptional activity of estrogen receptors ERα and ERβ in the E(1)C.1 cerebellar granule cell line. Brain Res 2007;1186:41–7. Epub 2007 Oct 23.
18. Omoto Y, Eguchi H, Yamamoto-Yamaguchi Y, Hayashi S. Estrogen receptor β1 and ERβcx/β2 inhibit ERα function differently in breast cancer cell line MCF7. Oncogene 2003;22:5011–20.
19. Sakamoto T, Eguchi H, Omoto Y, Ayabe T, Mori H, Hayashi S. Estrogen receptor-mediated effects of tamoxifen on human endometrial cancer cells. Mol Cell Endocrinol 2002;192:93–104.
20. Weinberg OK, Marquez-Garban DC, Fishbein MC, et al. Aromatase inhibitors in human lung cancer therapy. Cancer Res 2005;65:11287–91.
21. Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F, Pasqualini JR. Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. J Steroid Biochem Mol Biol 2000;72:23–7.
22. Suzuki T, Miki Y, Nakamura Y, et al. Sex steroid-producing enzymes in human breast cancer. Endocr Relat Cancer 2005;12:701–20.
23. Provost PR, Blomquist CH, Godin C, et al. Androgen formation and metabolism in the pulmonary epithelial cell line A549: expression of 17β-hydroxysteroid

dehydrogenase type 5 and 3α-hydroxysteroid dehydrogenase type 3. Endocrinology 2000;141:2786–94.
24. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996;93:5925–30.
25. Enmark E, Pelto-Huikko M, Grandien K, et al. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 1997;82:4258–65.
26. Korach KS. Insights from the study of animals lacking functional estrogen receptor. Science 1994;266:1524–7.
27. Kawai H, Ishii A, Washiya K, et al. Estrogen receptor α and β are prognostic factors in non-small cell lung cancer. Clin Cancer Res 2005;11:5084–9.
28. Wu CT, Chang YL, Shih JY, Lee YC. The significance of estrogen receptor β in 301 surgically treated non-small cell lung cancers. J Thorac Cardiovasc Surg 2005;130:979–86.
29. Dabbs DJ, Landreneau RJ, Liu Y, et al. Detection of estrogen receptor by immunohistochemistry in pulmonary adenocarcinoma. Ann Thorac Surg 2002;73:403–5; discussion 406.
30. Schwartz AG, Prysak GM, Murphy V, et al. Nuclear estrogen receptor β in lung cancer: expression and survival differences by sex. Clin Cancer Res 2005;11:7280–7.
31. Lau SK, Chu PG, Weiss LM. Immunohistochemical expression of estrogen receptor in pulmonary adenocarcinoma. Appl Immunohistochem Mol Morphol 2006;14:83–7.
32. Hershberger PA, Vasquez AC, Kanterewicz B, Land S, Siegfried JM, Nichols M. Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands. Cancer Res 2005;65:1598–605.
33. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983;31:13–20.
34. Vendrell JA, Bieche I, Desmetz C, et al. Molecular changes associated with the agonist activity of hydroxy-tamoxifen and the hyper-response to estradiol in hydroxy-tamoxifen-resistant breast cancer cell lines. Endocr Relat Cancer 2005;12:75–92.
35. Mah V, Seligson DB, Li A, et al. Aromatase expression predicts survival in women with early-stage non small cell lung cancer. Cancer Res 2007;67:10484–90.

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

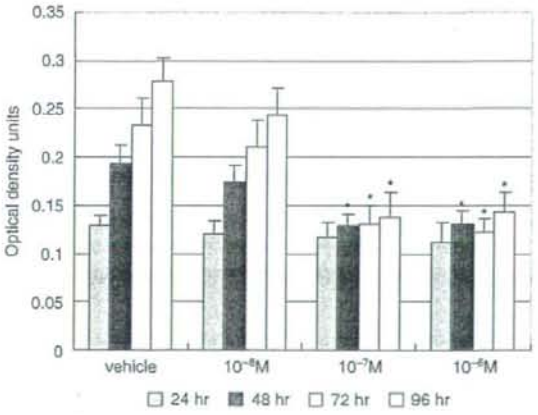

Fig. 1. Effect of AM580 on Ishikawa cell proliferation. Cells were treated with AM580 (10^{-8}–10^{-6} M) or with dimethylsulfoxide (DMSO; vehicle), as a control, for 24–96 h. Cell proliferation was analyzed in 96-well microplates by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric 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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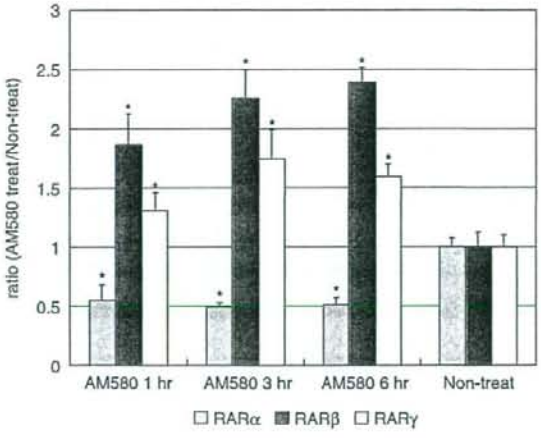

Fig. 2. Retinoic acid receptor (RAR) β and RARγ mRNA were induced by AM580 in Ishikawa cells. Real-time reverse transcription–polymerase chain reaction analysis was carried out to measure RAR mRNA expression in the presence or absence of AM580 treatment. Summary data for three independent experiments 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).

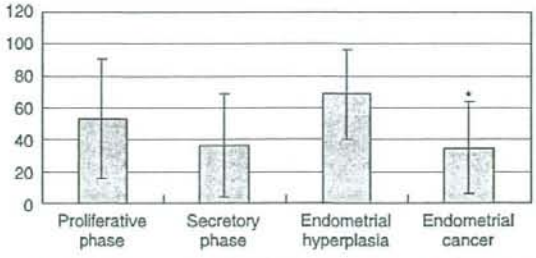

Fig. 4. Summary of immunohistochemistry for retinoic acid receptor (RAR) β. For evaluation of RARβ expression, we determined the labeling index (LI). Results are expressed as the mean ± SE. $^*P < 0.02$ versus endometrial hyperplasia.

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

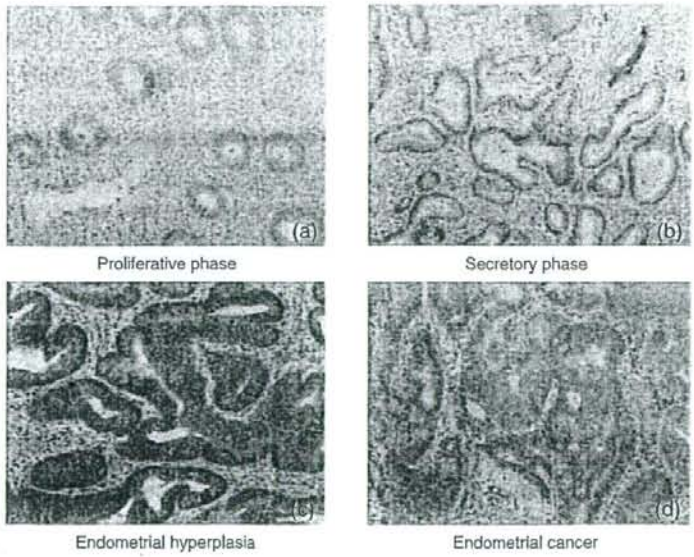

Fig. 3. Retinoic acid receptor (RAR) β localization in eutopic endometrium, endometrial hyperplasia, and endometrial carcinoma. (a,b) In ectopic endometrium, RARβ immunoreactivities were detected in the nuclei of epithelial cells throughout all menstrual phases. (c) In endometrial hyperplasia, RARβ immunoreactivity 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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References

1 Hong WK, Itri LM. Retinoids and human cancer. In: Sporn MB, Roberts AB, Goodman DS, eds. *The Retinoids: Biology, Chemistry and Medicine*, 2nd edn. New York: Raven Press, 1994; 597–630.
2 Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science* 1997; **278**: 1073–7.
3 Nason-Burchenal K, Dmitrovsky E. Retinoids in cancer therapy and chemoprevention. In: Bertino JR, ed. *Molecular Biology of Cancer*, 1st edn. San Diego: Academic Press, 1996; 1547–60.
4 Pfahl M, Piedrafita FJ. Retinoid targets for apoptosis induction. *Oncogene* 2003; **22**: 9058–62.
5 Pitha-Rowe I, Petty WJ, Kitareewan S, Dmitrovsky E. Retinoid target genes in acute promyelocytic leukemia. *Leukemia* 2003; **17**: 1723–30.
6 Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics. *CA Cancer J Clin* 1996; **46**: 5–27.
7 Kohler MF, Nishi H, Humphrey PA *et al.* Mutation of the p53 tumor-suppressor gene is not a feature of endometrial hyperplasias. *Am J Obstet Gynecol* 1993; **169**: 690–4.
8 Bo WJ, Smith MS. The effect of retinol and retinoic acid on the morphology of the rat uterus. *Anat Rec* 1996; **156**: 5–9.
9 Boettger-Tong HL, Stancel GM. Retinoic acid inhibits estrogen-induced uterine stromal and myometrial cell proliferation. *Endocrinology* 1995; **136**: 2975–83.
10 Loughney AD, Kumarendran MK, Thomass EJ, Redfern CPF. Variation in the expression of cellular retinoid binding proteins in human endometrium throughout the menstrual cycle. *Hum Reprod* 1995; **10**: 1297–304.
11 Kumarendran MK, Loughney AD, Prentice A, Thomas EJ, Redfern CP. Nuclear retinoid receptor expression in normal human endometrium throughout the menstrual cycle. *Mol Hum Reprod* 1996; **2**: 123–9.
12 Siddiqui NA, Loughney A, Thomas EJ, Dunlop W, Redfern CPF. Cellular retinoid binding proteins and nuclear retinoic acid receptors in endometrial epithelial cells. *Hum Reprod* 1994; **9**: 1410–16.
13 Arici A, Seli E, Senturk LM *et al.* Interleukin-8 in the human endometrium. *J Clin Endocrinol Metab* 1998; **83**: 1783–7.
14 FIGO. Stages-1988 revision. *Gynecol Oncol* 1989; **35**: 125–7.
15 Sasano H, Frost AR, Saitoh R *et al.* Aromatase and 17β-hydroxysteriod dehydrogenase type 1 in human breast carcinoma. *J Clin Endocrinol Metab* 1996; **81**: 4042–6.
16 Lotan R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim Biophys Acta* 1980; **605**: 33–91.
17 Morriss-kay G. Retinoic acid receptors in normal growth and development. *Cancer Surv* 1992; **14**: 181–93.
18 Lotan R, Xu XC, Lippman SM *et al.* Suppression of retinoic acid receptor-β in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med* 1995; **332**: 1405–10.
19 Xu XC, Sozzi G, Lee JS *et al.* Suppression of retinoic acid receptor β in non-small-cell lung cancer *in vivo*: implications for lung cancer development. *J Natl Cancer Inst* 1997; **89**: 624–9.
20 Picard E, Seguin C, Monhoven N *et al.* Expression of retinoid receptor genes and proteins in non-small-cell lung cancer. *J Natl Cancer Inst* 1999; **91**: 989–91.
21 Castillo L, Milano G, Santini J, Demard F, Pierrefite V. Analysis of retinoic acid receptor β expression in normal and malignant laryngeal mucosa by a sensitive and routine applicable reverse transcription–polymerase chain reaction enzyme-linked immunosorbent assay method. *Clin Cancer Res* 1997; **3**: 2137–42.
22 Widschwendter M, Berger J, Daxenbichler G *et al.* Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. *Cancer Res* 1997; **57**: 4158–61.
23 Qiu H, Zhang W, El-Naggar AK *et al.* Loss of retinoic acid receptor-β expression is an early event during esophageal carcinogenesis. *Am J Pathol* 1999; **155**: 1519–23.
24 Berg WJ, Nanus DM, Leung A *et al.* Up-regulation of retinoic acid receptor β expression in renal cancers *in vivo* correlates with response to 13-*cis*-retinoic acid and interferon-α-2a. *Clin Cancer Res* 1999; **5**: 1671–5.
25 Liu Y, Lee MO, Wang HG *et al.* Retinoic acid receptor β mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol Cell Biol* 1996; **16**: 1138–49.
26 Lippman FR, Lee JJ, Karp DD *et al.* Randomized phase III intergroup trial of isotretinoin to prevent second primary tumors in stage I non-small-cell lung cancer. *J Natl Cancer Inst* 2001; **93**: 605–18.
27 Khuri FR, Lee JJ, Lippman SM *et al.* Randomized phase III trial of low-dose isotretinoin for prevention of second primary tumors in stage I and II head and neck cancer patients. *J Natl Cancer Inst* 2006; **98**: 441–50.
28 Petty WJ, Li N, Biddle A *et al.* A novel retinoic acid receptor β isoform and retinoid resistance in lung carcinogenesis. *J Natl Cancer Inst* 2005; **97**: 1645–51.
29 Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R. Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Res* 1994; **54**: 3580–7.
30 Sabichi AL, Xu X, Lippman SM. RARβ1′: primed to fight retinoid resistance in lung carcinogenesis. *J Natl Cancer Inst* 2005; **97**: 1632–3.
31 Pitha-Rowe I, Petty WJ, Feng Q *et al.* Microarray analyses uncover *UBE1L* as a candidate target gene for lung cancer chemoprevention. *Cancer Res* 2004; **64**: 8109–15.
32 Freemantle SJ, Dragnev KH, Dmitrovsky E. The retinoic acid paradox in cancer chemoprevention. *J Natl Cancer Inst* 2006; **98**: 426–7.
33 Loughney AD, Redfern CP. Menstrual cycle related differences in the proliferative responses of cultured human endometrial stromal cells to retinoic acid. *J Reprod Fertil* 1995; **105**: 153–9.
34 Brar AK, Kessler CA, Meyer AJ, Cedars MI, Jikihara H. Retinoic acid suppresses *in vitro* decidualization of human endometrial stromal cells. *Mol Hum Reprod* 1996; **2**: 185–93.
35 Osteen KG, Keller NR, Feltus FA, Melner MH. Paracrine regulation of matrix metalloproteinase expression in the normal human endometrium. *Gynecol Obstet Invest* 1999; **48** (Suppl 1): 2–13.
36 Ito K, Suzuki T, Moriya T *et al.* Retinoid receptors in the human endometrium and its disorders: a possible modulator of 17β-hydroxysteroid dehydrogenase. *J Clin Endocrinol Metab* 2001; **86**: 2721–7.

Midkine and its clinical significance in endometrial carcinoma

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Midkine (MK) is a secreted heparin-binding growth factor. Several types of human cancer have increased MK expression with elevated serum levels. The purpose of this study was to determine whether MK was expressed in endometrial carcinoma and to evaluate the clinicopathological significance of serum MK in patients with endometrial carcinoma. Immunohistochemical expression of MK was evaluated in 85 endometrial carcinoma samples and 33 controls. MK expression was significantly higher in the carcinomas than in normal endometrium ($P < 0.001$). Interestingly, MK expression was highest at the margins of invasion and low in the superficial areas of the tumor samples. Using ELISA, we compared serum MK concentration in 120 endometrial carcinoma patients with the concentration in 46 patients with benign gynecologic tumors. Serum MK value in patients with cancer was significantly higher than that in the patients with benign diseases ($P = 0.01$). Patients with positive lymph node metastasis or recurrence, or cancer death, had a higher serum MK level ($P = 0.008$, $P = 0.009$, respectively). In conclusion, MK immunoreactivity in endometrial carcinoma is significantly higher than in normal endometrium. Additionally, preoperative serum MK levels are significantly correlated with prognosis and the presence of lymph node metastasis. Thus, MK may be a useful serum biomarker for identifying high risk patients of endometrial carcinoma. (*Cancer Sci* 2008; 99: 1125–1130)

Endometrial carcinoma is one of the most common female pelvic malignancies worldwide, and its incidence has recently increased in Japan.[1,2] As approximately 80% of endometrial carcinomas are diagnosed at an early stage when surgery is curative, they carry a better prognosis than other cancers. However, advanced or recurrent cases tend to respond poorly to conventional treatments such as radiation, chemotherapy, or hormonal therapy, and as a result carry a poor prognosis. Identification of additional prognostic markers could help detect patients at a high risk of relapse or death from the disease.

Clinical, biological, and epidemiological findings all suggest that prolonged or unopposed estrogenic stimulation increases the risk of type I endometrial carcinoma. The initiation and progression of type I endometrial carcinoma, however, are poorly understood at a molecular level. We previously studied the gene expression profile of endometrioid adenocarcinoma, and identified 24 genes that had at least a 1.5-fold increased expression in both well (grade 1) and poorly (grade 3) differentiated endometrioid adenocarcinoma compared to normal endometrium (unpublished data). MK was identified as one of the up-regulated genes. Though MK expression has been reported in many human cancers, it has not been studied in endometrial carcinoma. Therefore, we focused our subsequent experiments on the actions of MK.

MK is a secreted, heparin-binding growth factor. It is a 13-kDa protein rich in basic amino acids and cysteine.[3,4] MK is highly expressed in the mid-gestational period during embryogenesis, and is involved in tooth, lung, kidney, and bone development. In the adult, MK has a very restricted pattern of expression. The highest transcript levels are in the intestine with low levels in the cerebellum, thyroid, kidney, bladder, lung alveoli, colon, stomach, and spleen.[5] The pathophysiological effects of MK include the oncogenic transformation of fibroblasts, antiapoptotic activity, and angiogenic activity.[6–8] MK mRNA levels and protein expression are frequently elevated in various human carcinomas of the breast, lung, esophagus, colon, ovary, urinary bladder, and prostate; and glioblastomas, neuroblastomas, and Wilms' tumor.[9–18] Furthermore, MK concentrations in serum are also elevated in various carcinomas.[19–22] To our knowledge, however, no study has focused on the clinicopathological significance of MK expression in human endometrial carcinoma. The purpose of this study was to determine whether MK was expressed in endometrial carcinoma, and whether differences existed between the expression level in cancer and levels in benign gynecologic conditions. We also explored whether correlations existed between MK expression and clinicopathological features.

Materials and Methods

Tissue and serum samples. Eighty-five endometrioid endometrial carcinomas (37 well differentiated, 25 moderately differentiated, 23 poorly differentiated; 55 stage I, 16 stage II, 11 stage III, three stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan for immunohistochemical analysis. The controls were selected from patients who underwent hysterectomy for benign gynecologic diseases without any personal cancer history from April 1996 to March 2004. The median follow-up time for patients whose samples were examined immunohistochemically was 60 months (range, 2–148 months). The disease-free and overall survival times of the patients were calculated from the time of initial surgery to recurrence or death, or the date of last contact. The survival times of patients still alive or lost to follow-up were censored in December 2004. Serum samples were obtained from 120 patients with endometrial carcinoma (66 well differentiated, 16 moderately differentiated, 12 poorly differentiated, 26 other histological type; 80 stage I, 11 stage II, 17 stage III, 12 stage IV) and from 45 patients with non-malignant gynecologic diseases at Tohoku University Hospital from April 2002 to January 2007. None of the patients examined had received radiation, hormonal therapy, or chemotherapy prior to surgery. The median follow-up time for the patients whose serum was tested for MK was 91 months (range, 1–166 months). The

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survival times of patients still alive or lost to follow-up were censored in August 2007. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine.

Total RNA extraction from endometrial tissues and cDNA synthesis. All tumor and normal specimens were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from normal endometrium and carcinoma tissues, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A reverse transcription kit, SuperScript III RT (Invitrogen, Carlsbad, CA, USA), was used for the synthesis of cDNA.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). Real-time PCR was carried out using the LightCycler System (Roche Diagnostics, Mannheim, Germany). cDNAs of known concentrations for target genes and the housekeeping gene, ribosomal protein L13a (RPL13A) were used to generate standard curves for determining the quantity of target cDNA transcripts. The mRNA level in each case was represented as a ratio with RPL13A.[23] The PCR thermal profile for MK was: initial denaturation at 95°C for 10 min followed by 32 amplification cycles of denaturation at 95°C for 10 s, annealing at 68°C for 10 s, and elongation at 72°C for 12 s; and for RPL13A, initial denaturation at 95°C for 10 min followed by 30 amplification cycles of denaturation at 95°C for 12 s, annealing at 68°C for 10 s, and elongation at 72°C for 12 s.

The primer sequences used in our study were: 5′-CCA AGA CCA AAG CAA AGG-3 and 5′-GGC AGG GCA TGA TTG ATT-3′ for MK; 5′-CCT GGA GGA GAA GAG GAA AGA GA-3′ and 5′-TTG AGG ACC TCT GTG TAT TTG TCA A-3′ for RPL13A.

Immunohistochemistry. After deparaffinization and rehydration in graded alcohol, antigen retrieval for MK immunostaining was done by heating the sections in a 600-W microwave for 20 min in 10 mM trisodium citrate buffer, pH 7.0. The sections were then blocked with normal goat serum for 30 min at room temperature, followed by incubation with chicken antihuman MK antibody (given by K.K.) overnight at 4°C. The dilution of the primary antibody used in this study was 1/250. The slides were incubated in 99.7% methanol containing 0.3% hydrogen peroxide at room temperature for 30 min to inhibit endogenous peroxidase. They were then incubated with biotin-conjugated rabbit antichicken IgG (ICN Pharmaceuticals, Aurora, OH, USA) at room temperature for 30 min, followed by incubation with peroxidase-conjugated streptavidin for 30 min at room temperature, using a Histofine Kit (Nichirei, Tokyo, Japan). The antigen-antibody complex was visualized with a 3, 3′-diaminobenzidine solution (1 mmol/L 3, 3′-diaminobenzidine, 50 mmol/L Tris-HCl [pH 7.6], 0.006% H2O2) and counter-stained with hematoxylin. Serous adenocarcinoma of the ovary was employed as a positive control for MK immunostaining.[13] The primary antibody was replaced with phosphate-buffered saline (PBS) as a negative control. Samples were considered negative if none of the cells stained for MK. Very weak positive was defined as less than 5% staining, weak positive as 5–25% staining, moderate positive as 25–50% staining, and strong positive as more than 50% staining. Slides were then numerically scored based on immunoreactivity. A score of 0 was negative, 1 very weak, 2 weak, 3 moderate, and 4 strong positive.

ELISA for human MK. An ELISA for human MK was performed as described previously.[22] Briefly, human MK was produced using Pichia pastoris GS115 by transfection with a human MK expression vector, which was constructed into pHIL-D4 (Invitrogen). This yeast-produced human MK was used to immunize rabbits and chickens to raise antibodies. The rabbit antihuman MK antibody (50 mL of 5.5 mg/mL in 50 mM Tris HCl [pH 8.2], 0.15 M NaCl, 0.1% NaN_3) was coated onto the wells of microtiter plates (Polysorpplates; Nunc, Rochester, NY, USA) for 20 h at room temperature. After washing with 0.05% Tween 20 in PBS, the wells were blocked with 300 mL of 0.1% casein, 0.01% Microcide I (aMReSCO) in PBS for 20 h at 37°C. Plasma samples (10 mL each) were mixed with 100 mL of 50 mM Tris HCl (pH 8.4), 0.5 M KCl, 0.1% casein, 0.5% bovine serum albumin, 0.01% Microcide I, and 0.1 mg/mL peroxidase-labeled chicken antihuman MK antibody. Aliquots of 50 mL of this mixture were added to wells prepared as described above, and subjected to chromogenic detection at OD450 using tetramethylbenzidine as the substrate. This ELISA system shows linearity from 0 to 4 ng/mL of MK, and there is no crossreaction with Pleiotrophin.[22]

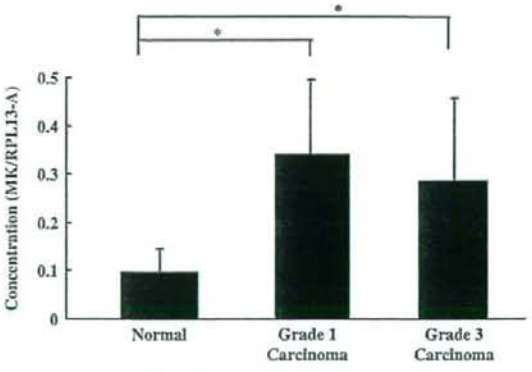

Fig. 1. Midkine (MK) mRNA expression levels in normal endometrial tissues and endometrial carcinoma tissues measured by reverse transcription–polymerase chain reaction (RT-PCR). MK mRNA expression levels in carcinoma tissues were significantly higher than in normal endometrial tissues ($P < 0.001$, Mann–Whitney test).

Statistical analysis. mRNA levels and serum concentrations of MK were compared using the Mann–Whitney test. Immunoreactivities for MK were compared using a Student's *t*-test. *P*-values less than 0.05 were considered significant.

Results

MK was expressed at higher levels in endometrioid adenocarcinoma tissues than in normal endometrium samples. To validate the microarray-based MK expression difference, we performed real-time RT-PCR using cDNA from 10 normal endometrium specimens and 20 carcinoma specimens; 10 were grade 1 and 10 were grade 3. The quantitative mRNA expression levels of MK were significantly higher in the endometrioid adenocarcinomas than in normal endometrium samples. However, there was no difference in the expression level between grade 1 and grade 3 (Fig. 1).

We then confirmed the high expression of MK in carcinoma tissues not only at the mRNA level but also at the protein level by immunohistochemical staining. The intensity of MK immunostaining in tissues is summarized in Table 1. As shown in Figure 2, MK protein was predominantly expressed in the epithelial cytoplasm with little nuclear expression. Positive staining for MK was scarcely detected in the stroma. In both normal proliferative and secretory phase endometrium samples, MK expression in the basal layer was significantly stronger than in the functional layer or endometrial stroma ($P < 0.001$, *t*-test) (Table 1 and Fig. 2c–f). No significant difference in protein expression was detected between the endometrial stroma and the functional layer in either the proliferative or the secretory phase. MK immunoreactivity at the basal layer tended to be stronger in

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Table 1. Midkine protein expression in normal and endometrial cancer tissues by immunohistochemistry (mean ± SD of immunostaining score)

Normal	*n*	Endometrial stroma	Functionalis	Basalis	
Total	33	0.41 ± 0.56	0.62 ± 0.89	*1.72 ± 1.17*	*P* < 0.001*
Proliferative	21	0.35 ± 0.61	0.62 ± 0.86	1.35 ± 1.18	
Secretory	12	0.50 ± 0.52	0.67 ± 0.98	2.17 ± 0.94	
Carcinoma	*n*	Endometrial stroma	Superficial area	Invasive area	
Total	85	0.38 ± 0.56	0.81 ± 0.78	*2.66 ± 0.79*	
G1	37	0.41 ± 0.55	1.00 ± 0.77	2.69 ± 0.82	
G2	25	0.32 ± 0.56	0.60 ± 0.71	2.56 ± 0.77	
G3	23	0.39 ± 0.58	0.75 ± 0.79	2.74 ± 0.81	

*P-value, *t*-test.

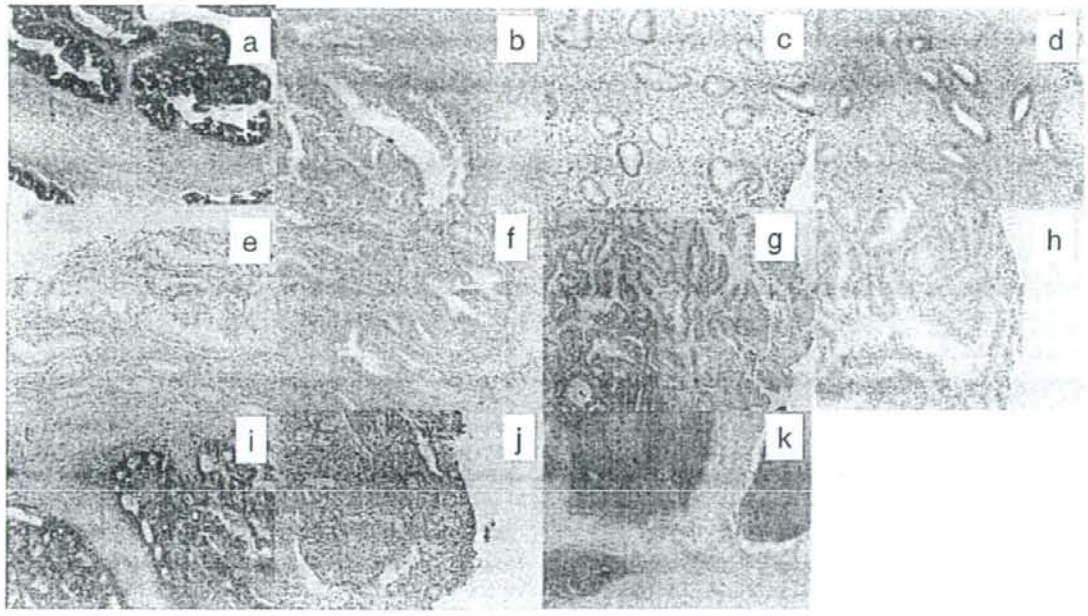

Fig. 2. Representative panels of immunohistochemical staining with anti-midkine (MK) protein antibody. (a) Positive control, (b) negative control, (c) proliferative phase (functionalis), (d) proliferative phase (basalis), (e) secretory phase (functionalis), (f) secretory phase (basalis), (g) transitional area of endometrial carcinoma grade 1, (h) superficial area of endometrial carcinoma grade 1, (i) invasive area of endometrial carcinoma grade 1, (j) superficial area of endometrial carcinoma grade3, (k) invasive area of endometrial carcinoma grade 3.

the secretory phase than in the proliferative phase ($P = 0.09$, *t*-test). Interestingly, MK expression was strongest at the margins of invasion and low in the superficial layers of the tumor samples (Fig. 2g–j). MK expression was significantly higher in the carcinomas than in the basal area of the normal endometrium ($P < 0.001$, *t*-test) (Table 1). No statistical correlation was detected between grade 1 and grade 3 endometrioid adenocarcinoma. MK immunoreactivity was not associated with any clinicopathological features including histological grade, depth of myometrial invasion, the presence of lymph node metastasis, or prognosis.

Serum MK protein concentration was higher in patients with endometrial carcinoma than in patients with benign gynecologic diseases. We measured serum MK protein concentrations with ELISA. Serum MK values for the patients with endometrial carcinoma was significantly higher than those for patients with benign gynecologic diseases ($P = 0.01$, Mann–Whitney test). The data suggest that MK protein is not only expressed in cancer tissues but also secreted into the sera at higher levels in endometrial carcinoma patients. To test whether the serum MK level could be used to discriminate endometrial carcinoma from benign disease, we set various cut-off values and classified the cases based on their MK values. Serum MK level had a high false negative ratio, thereby limiting its use in clinical applications.

A higher serum MK protein concentration was correlated with the presence of lymph node metastases and prognosis of endometrial carcinomas. We calculated the mean serum MK concentrations of cancer patients categorized by clinicopathological features. Results of the associations between clinicopathological parameters and serum MK levels are summarized in Table 2. Serum MK concentration was not associated with age, histological grade, or lymphovascular invasion. Although serum MK had a tendency to be lower in stage I–II or no myometrial

Table 2. Serum midkine (MK) levels and clinicopathological factors in endometrial carcinomas

Clinicopathological factors		*N* (%)	MK concentrations (Mean ± SD)	*P**-values
Age	50 =	25 (21)	104 ± 253	0.111
	50 <	95 (79)	81 ± 113	
Histological grade	Grade1	66 (55)	82 ± 169	0.455
	Grade2	16 (13)	64 ± 97	
	Grade3	12 (10)	144 ± 112	
	Others	26 (22)	76 ± 143	
Stage	I–II	91 (76)	71 ± 157	0.054
	III–IV	29 (24)	133 ± 159	
Myometrial invasion	None	19 (16)	46 ± 76	0.074
	< 1/2	58 (48)	79 ± 178	
	= 1/2	40 (33)	100 ± 130	
	Unknown	5 (4)	183 ± 201	
Lymphovascular invasion	Negative	83 (69)	75 ± 153	0.720
	Positive	35 (29)	90 ± 139	
	Unknown	2 (2)	400 ± 33	
Lymph node metastasis	Negative	103 (86)	73 ± 142	0.008
	Positive	5 (4)	253 ± 246	
	Unknown	12 (10)	131 ± 161	
Prognosis	Non-recurrence	102 (85)	71 ± 142	0.009
	Recurrence or death	18 (15)	172 ± 184	

**P*-value, Mann–Whitney test.

invasion, the difference was not statistically significant ($P = 0.054$, $P = 0.072$). Interestingly, the patient group with positive lymph node metastasis had a higher level of serum MK ($P = 0.008$, Mann–Whitney test). Patients with recurrence or cancer related death had significantly higher serum levels of MK protein than those without recurrence ($P = 0.009$).

Discussion

This is the first report showing that mRNA levels and protein expression of MK in endometrial carcinoma are significantly higher than in normal endometrium. Additionally, serum MK levels in endometrial carcinoma patients were significantly elevated relative to levels in patients with benign gynecologic diseases. Although MK is overexpressed in various human malignant tumors, its effects on tumor growth and progression are not fully understood. Growth of mouse colorectal carcinoma cells is inhibited by antisense midkine oligo DNA.[24] Transfection of the breast carcinoma line MCF-7 with MK accelerates tumor growth and increases tumor vascularity after cell implantation in nude mice.[25] MK also rescues Wilms' tumor cells from cisplatin-induced apoptosis.[26] These effects are likely mediated by signaling via phosphatidylinositol-3-kinase and mitogen-activated kinase.[27] Taken together these biological data support the hypothesis that MK plays an important role in oncogenesis and tumor progression.

Despite the increased MK immunoreactivity in endometrial carcinomas, there was no relationship between immunoreactivity and clinicopathological features. This was surprising since high MK immunoreactivity significantly correlates with worse clinical outcome of neuroblastomas,[17] urinary bladder cancer,[14] gastrointestinal stromal tumor,[28] oral squamous cell carcinomas,[29] and pancreatic cancer.[30] Interestingly, in esophageal carcinoma, MK is more intensely expressed in well-differentiated tumors than in poorly differentiated tumors.[11] A noteworthy immunohistochemical finding in this study was that the intensity of MK protein expression was not the same across different areas within a single tissue sample. MK expression in normal endometrium was higher in the basalis than in the functionalis. It was highly expressed at the margin of invasion but not in the superficial areas of the cancer specimens. To confirm that these findings were not due to the unequal localization of antibody, endometrial biopsy samples from cancer patients were also immunostained. These superficial specimens all demonstrated weak expression (data not shown). The MK immunohistochemical findings in normal endometrium were inconsistent with the previously reported pathophysiological effects of MK. MK is involved in angiogenesis and antiapoptosis. Microvessel density in normal endometrium, however, is not significantly different between the functionalis and basalis,[31] and apoptotic cells are equally distributed on each layer.[32] Donoghue *et al.* reported that lymphatic vessel density (LVD) is higher in the basalis than in the functionalis across the menstrual cycle.[33] In this study, the distribution of lymphatic vessels is consistent with the diversity of MK immunoreactivity across the menstrual cycle. Rogers *et al.* suggested that unknown lymphangiogenic growth factors may be involved in normal endometrium, since no difference is observed in immunostaining intensity for the vascular endothelial growth factor (VEGF)-C or VEGF-D between the functionalis and basalis.[33] We speculate that MK would be a candidate molecule for lymphangiogenesis in normal endometrium. In endometrial adenocarcinoma, the peritumoral LVD is higher compared with the LVD within the tumor and in normal endometrium, which also correspond to MK immunoreactivity. These observations suggest a role for MK in lymphangiogenesis in endometrial adenocarcinoma.

Since MK is a secretory protein, it could potentially be used to screen for and monitor the progression of endometrial carcinoma in a manner similar to cancer antigen (CA)–125 for ovarian cancer. An elevated serum MK level is detected in more than 80% of human adult carcinomas, and its level decreases when the tumor is resected.[19] A high serum MK level is associated with higher stage and disease progression in gastric cancer,[21] with tumor size in esophageal cancer,[20] and with progression in neuroblastoma.[22] As shown in Figure 3, serum MK was significantly elevated in patients with endometrial carcinoma compared with patients with non-malignant gynecologic diseases ($P = 0.014$). Regarding the relationship between serum MK concentration and clinicopathological features in patients with endometrial carcinoma, statistical differences were seen in both lymph node metastasis and prognosis. Our observations are consistent with another recent study in esophageal carcinoma.[34] In

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