Orphan nuclear receptor DAX-1 in human endometrium and its disorders

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(Received March 7, 2005/Revised August 1, 2005/Accepted August 1, 2005/Online publication October 3, 2005)

DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1) is a recently characterized member of the orphan nuclear receptor family. DAX-1 functions as a global negative regulator of steroid hormone production. It inhibits adrenal 4 binding protein (Ad4BP)/steroidogenic factor-1 (SF-1) pathway-dependent P450arom expression in cultured human endometriotic stromal cells and acts as a corepressor for estrogen receptors (ER). In this study we first examined the localization of DAX-1 in 46 normal cycling endometria, 36 cases of endometrial hyperplasia and 103 cases of endometrial carcinoma by using immunohistochemistry to elucidate the possible involvement of DAX-1 and its correlation to the status of Ad4BP/SF-1, a universal transcription factor of steroidogenesis. We then evaluated DAX-1 mRNA expression, using quantitative reverse transcription–polymerase chain reaction for DAX-1 in 33 cases of endometrial carcinoma for further characterization. We subsequently correlated these findings with various clinicopathological parameters of the cases. Ad4BP/SF-1 immunoreactivity was not detected in any human endometria examined. A significant inverse correlation was detected between the status of DAX-1 immunoreactivity and histological grade ($P = 0.0003$) in endometrial carcinoma. The labeling index (LI) values of DAX-1 in normal endometrium during the secretory phase ($P < 0.0001$) and hyperplasia ($P < 0.0001$) were significantly higher than that of carcinoma. No significant correlations were detected between DAX-1 immunoreactivity and amounts of aromatase mRNA. There was a statistically significant positive correlation between DAX-1 and ERα ($P = 0.006$) and ERβ LI ($P < 0.001$). These findings suggest that DAX-1 may inhibit the proliferation and progression of endometrial carcinoma through inhibition of estrogenic actions, possibly by interacting with ER present in carcinoma cells, rather than regulating *in situ* steroidogenesis. (*Cancer Sci* 2005; 96: 645–652)

Endometrial carcinoma is one of the most common pelvic malignancies among women worldwide, and its incidence has recently increased.[1,2] *In situ* estrogen metabolism, including synthesis and degradation, has been recently recognized as a very important factor in the development and progression of various human estrogen-dependent neoplasms, including endometrial carcinoma, especially the endometrioid type.[3,4] In endometrial carcinoma, *in situ* 17β-estradiol (E2) availability has been reported to be closely associated with the pathogenesis and development of endometrial proliferative disorders including endometrial hyperplasia and carcinoma, especially the endometrioid type.[5]

DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1) is a recently characterized member of the orphan nuclear receptor family.[6,7] DAX-1 is expressed in the adrenal cortex, ovary, Leydig cells and other endocrine cells such as testicular Sertoli cells, pituitary gonadotropes, ventromedial hypothalamic nucleus cells and others.[8,9] DAX-1 acts as a negative regulator of steroid production by repressing the expression of steroidogenic acute regulatory protein (StAR) in the adrenal cortex and gonads,[10–12] side-chain cleavage P450 (P450scc) and 3β-hydoroxysteroid dehydrogenase (3β-HSD).[13] DAX-1 has also been demonstrated to repress Ad4BP/SF-1-mediated transactivation of other steroidogenic genes,[14,15] including genes for P450c17 and aromatase p450 (P450arom).[16–18] DAX-1 inhibits Ad4BP/SF-1 pathway-dependent P450arom expression in cultured human endometriotic and endometrial stromal cells.[19] In addition, DAX-1 interacts with both subtypes of estrogen receptors, ERγ and ERβ. This interaction occurs with ligand-activated ER, and is mediated by a unique DAX-1 N-terminal repeat domain.[6,11,20] DAX-1 itself also acts as a corepressor for ER. These interactions could play significant roles by influencing estrogen signaling pathways, because of the reported coexpression of DAX and ER in reproductive tissues.[21] However, the expression of DAX-1 and Ad4BP/SF-1 has not been reported in the human endometrium and its disorders. Therefore, their possible roles as transcription factors of steroidogenic enzymes in the human endometrium and its disorders still remain unknown. In this study, we examined the status of DAX-1 and Ad4BP/SF-1 in endometrial carcinoma, endometrial hyperplasia and normal endometrium, and correlated the findings with the status of aromatase, ERγ, ERβ, progesterone receptor (PR) and Ki67 in order to examine the possible biological significance of this unique transcription factor in both physiological and pathological human endometrium.

Materials and Methods

Tissue preparation

Forty-six normal cycling human endometria (26 proliferative phase, 20 secretory phase), 36 endometrial hyperplasias

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 doi: 10.1111/j.1349-7006.2005.00101.x

(16 simple hyperplasia, nine complex hyperplasia, and 11 atypical complex hyperplasia), and 103 endometrial endometrioid adenocarcinomas (49 well differentiated, 32 moderately differentiated, 22 poorly differentiated; 67 stage I, 12 stage II, 21 stage III, four stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). Non-pathological endometrium specimens were obtained from hysterectomies performed due to *in situ* carcinomas of the uterine cervix. Atypical endometrial complex hyperplasia and carcinoma specimens were obtained from hysterectomies. Simple hyperplasia and complex hyperplasia specimens were obtained from total dilatation and curettage. None of the patients examined had received irradiation, hormonal therapy or chemotherapy prior to surgery. The histopathological classification in each specimen was evaluated according to the International Federation of Gynecology and Obstetrics histological grading system for endometrial carcinoma proposed in 1988.[22] Human adrenal glands were retrieved from autopsy files, and breast carcinomas were obtained from surgical pathology. The specimens were all routinely processed (i.e. fixed in 10% formalin for 24–48 h), embedded in paraffin, and thin sectioned (3 μm).

Cell lines and media

The cervical carcinoma cell line HeLa and the adrenal gland cell line H295R were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KS, USA) and 1% penicillin/streptomycin. HeLa and H295R were used as positive controls for immunoblotting of DAX-1.

Primary antibodies

Rabbit polyclonal antibody for DAX-1 was obtained from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). An application of this antibody in immunohistochemistry investigation has been previously reported.[23–28] The polyclonal antibody for Ad4BP/SF-1 was provided by Dr K Morohashi (National Institute for Basic Biology, Okazaki, Japan). The generation and characterization of Ad4BP/SF-1 antibody has been described previously,[29] and the application of this antibody in an immunohistochemical study has also been reported previously.[30,31] Monoclonal antibodies for ERγ, ERβ, PR and Ki67 were purchased from Novocastra, Genetex, Chemicon (Temecula, CA, USA), and Dako Cytomation, respectively.

Immunohistochemistry

Immunohistochemical analyses were conducted with the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). After deparaffinization, antigen retrieval for DAX-1, Ad4BP/SF-1, ERγ, ERβ, PR and Ki67 analyses was performed by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate; pH 6.0). The dilutions of the primary antibodies used in our study were as follows: DAX-1, 1/500; Ad4BP/SF-1, 1/1000; ERγ, 1/50; ERβ, 1/1500; PR, 1/50; and Ki67, 1/50. 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 adrenal gland were used as positive controls for DAX-1 and Ad4BP/SF-1, and tissue sections of breast cancer were also used as positive controls for ERγ and ERβ. For negative controls, normal rabbit or mouse IgG was used instead of primary antibodies. We also made sections that were incubated with DAX-1 preabsorbed with synthetic antigen peptide (10 μg/mL) as primary antibody, for use as negative controls for immunostaining. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity

Evaluation of DAX-1, Ad4BP/SF-1, ERγ, ERβ, PR and Ki67 immunoreactivity was performed in high-power fields (×−400) using a standard light microscope. Two of the authors (SS and HU) simultaneously searched the entire tissue sections and determined the most representative areas using a double-headed light microscope. In all cases examined, a total of more than 500 tumor cells from three different representative fields were counted independently by the two aforementioned authors, and the percentage immunoreactivity (i.e. labeling index [LI]), was determined. After completely reviewing the immunostained sections of each lesion, two of the authors (SS and HU) independently divided the cases into the following two groups: + (more than 10% positive cells); – (fewer than 10% positive cells). Layfield *et al*. proposed the separation of ER- or PR-positive cases using LI cut-off points of 10% in the immunohistochemical analysis of human breast cancer.[32] Therefore, in this study, we used the same LI cut-off point between positive and negative DAX-1 of 10%. Cases with discordant results (interobserver differences of more than 5%) were simultaneously reevaluated by the same authors using a double-headed light microscope. Inter-observer differences were less than 5% in this study.

Reverse transcription–polymerase chain reaction

Thirty-three specimens of fresh-frozen (i.e. specimens immediately frozen in liquid nitrogen and stored at −80°C) endometrial carcinoma were available for examination. Total RNA was extracted by homogenizing frozen tissue samples in 1 mL TRIzol reagent (Life Technologies, Grand Island, NY, USA), followed by phenol-chloroform phase extraction and isopropanol precipitation. All RNA samples were quantified by spectrophotometry and stored at −80°C until processed for reverse transcription. Total RNA (4 μg) was denatured at 70°C for 10 min and was reverse transcribed in the presence of 50 ng/μL oligo (deoxythymidine) primer (Invitrogen Corporation, CA, USA), 2.5 mmol/L $MgCl_2$, 0.5 mmo/L deoxy-NTPs, 10 mmol/L dithiothreitol and 10 U ribonuclease H-reversed transcriptase (Superscript II RT, Invitrogen Corporation) for 60 min at 42°C and 15 min at 70°C on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research, Watertown, MA, USA). Following reverse transcription–polymerase chain reaction (RT–PCR), analysis was performed in order to examine the presence or absence of genomic DNA contamination. The RT step was performed in the absence of Superscript II RNase H^- Reverse Transcriptase, followed by PCR. RT–PCR products lacking reverse transcriptase in the

 doi: 10.1111/j.1349-7006.2005.00101.x

initial RT step were run on an ethidium-bromide-stained 2% agarose gel. No bands were detected in these samples (data not shown). The resulting cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the Light Cycler System (Roche Diagnostics, Mannheim, Germany) using the DNA-binding dye Syber Green I (Roche Diagnostics). The 20-μL reaction mixture contained 3 mM $MgCl_2$ for the DAX-1 and aromatase primer, 2 mM $MgCl_2$ for the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) primer, 10 pmol/L of each primer (Table 2) and the DNA-binding dye LightCycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics). GAPDH expression was used to verify the integrity of RNA from each specimen. Human gene specific primers used to amplify DAX-1 for PCR were: 5′-CCAAGCCATCAAGTGCTTTC-3′ (sense primer, 5′ position at 1308 bp), 5′-ATTTGCTGAGTTCCCCACTG-3′ (antisense primer, 5′ position at 1463 bp), which amplifies a PCR fragment of 156 bp; primers for aromatase were 5′-GTGAAAAAGGGGACAAACAT-3′ (sense primer, 5′ position at 1286 bp), 5′-TGGAATCGTCTCAGAAGTGT-3′ (antisense primer, 5′position at 1500 bp), generating a PCR fragment of 215 bp; primers for Ad4BP/SF1were 5′-GCAGAAGAA-GGCACAGATTC-3′ (sense primer, 5′ position at 439 bp), 5′-TGGGTACTCAGACTTGATGG-3′ (antisense primer, 5′ position at 713 bp), generating a PCR fragment of 274 bp; primers for GAPDH were 5′-TGAACGGGAAGCTCACTGG-3′ (sense primer, 5′ position at 731 bp), 5′-TCCACCACCCT-GTTGCTGTA-3′ (antisense primer, 5′ position at 1037 bp), generating a PCR fragment of 307 bp. An initial denaturing step of 95°C for 10 min was followed by 35 cycles, respectively, of 95°C for 15 s; 10 s annealing at 67°C (DAX-1), 60°C (aromatase and GAPDH) or 56°C (Ad4BP/SF-1); and extension for 13 s at 72°C. The fluorescence intensity of the double-stranded specific Syber Green I, which reflects the amount of specific PCR products formed, was read by the LightCycler at 85°C after the end of each extension step.[33] Using the automated programs of the LightCycler software, the amount of DAX-1, aromatase and GAPDH templates in each sample were calculated so as to be equal to the dilution of standard cDNA. The actual values of DAX-1 and aromatase template in each sample were corrected with the value of GAPDH template. Although conventional quantitative PCR requires the use of purified plasma cDNA in the construction of a standard curve, it was possible to semiquantify PCR products with the LightCycler using purified cDNA of known concentrations.[34,35]

Following PCR, these products were resolved on a 2% agarose ethidium bromide gel and examined under ultraviolet light to confirm the presence of a band of the anticipated size. In the initial experiments, PCR products were purified and subjected to direct sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 310 Genetic Analyzer, Perkin-Elmer, PE Applied Biosystems, Foster City, CA, USA) to verify amplification of the correct sequences. As positive controls, frozen adrenal gland tissues were used for DAX-1 and Ad4BP/SF-1, and full-term placenta was used for aromatase. Negative control experiments lacked the cDNA substrate to check for the presence of exogenous contamination of DNA. No amplified products were observed under these conditions.

Immunoblotting

Cells were grown to 70% confluence in 10-cm plates, and after removal of culture medium with phosphate-buffered saline (PBS), protein was extracted by using conventional methods. The protein concentration was measured by using a microplate reader (model 680, Biorad, Hercules, CA, USA) using Bradford reagent (Biorad). In all, 70 μg of protein of each sample was mixed with an equal volume of 2× concentrated sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresed (SDS-PAGE) sample buffer, boiled, and then transferred to nitrocellulose membrane (Hybond PDVF, Biorad). The reacted membranes were incubated in a blocking solution (PBS containing 3% non-fat milk and 0.1% Tween-20) for 18 h at 4°C, and then incubated in a 1:200 dilution of DAX-1 antibody in PBS (containing 0.1% Tween-20) for 60 min. After incubation with horseradish peroxidase-labeled anti rabbit immunoglobulin G, the antigen–antibody complex was subsequently reacted with the enhanced chemiluminescence system (Amersham, Germany). The specificity of the DAX-1 antibody was demonstrated by using an immunoabsorption test on preabsorption of the antibody with a synthetic peptide.

mRNA *in situ* hybridization

We further established the specificity of the DAX-1 antibody using mRNA *in situ* hybridization of DAX-1. Adrenal gland tissue specimens were used for this confirmation. mRNA *in situ* hybridization was performed by using the Discovery system (Ventana Medical Systems) as previously described.[36] The sequence of the 50-base antisense oligodeoxynucleotide probes for DAX-1 used for mRNA *in situ* hybridization was as follows: 5′-TTCGATGAATCTGTCATGGGGCCCTTGGT-GCGTCATCCTGGTGTGTTCAC-3′, which corresponds to nucleotides 1469–1518. A corresponding sense oligonucleotide probe was used as a negative control. The sequence of this sense probe was as follows: 5′-GTGAACACACCAGGAT-GACGCACCAAGGGCCCCATGACAGATTCATCGAA-3′. Tissue slides were loaded onto the Discovery automated slide-processing system. The baking and deparaffinization steps were performed as programmed in the protocol for the RiboMap *in situ* hybridization regent system (Ventana Medical Systems) on the instrument. After the deparaffinization step, the protocols for mRNA *in situ* hybridization were designed based on the standard protocols of the RiboMapKit (Ventana Medical Systems). The first fixation step was performed using the formalin-based RiboPrep reagent (Ventana Medical Systems) for 30 min at 37°C. Fixed tissue sections were acid-treated using the hydrochloride-based RiboClear reagent (Ventana Medical Systems) for 10 min at 37°C. The slides were subsequently incubated for protease digestion using protease 2 (Ventana Medical Systems) for 2 min at 37°C. After a denaturing step for 10 min at 70°C, the incubated tissue slides were reacted for hybridization with DAX-1 (200 ng/slide), antisense or sense RNA probe diluted with RiboHybe hybridization buffer (Ventana Medical Systems) for 6 h at 65°C. Hybridized tissue sections were washed in three stringency steps using 0.1× RiboWash (Ventana Medical Systems) for 6 min each at 65°C. The second fixation step was performed using RiboPrep reagent for 20 min at 37°C, followed by incubation with biotin-labeled antidigoxigenin

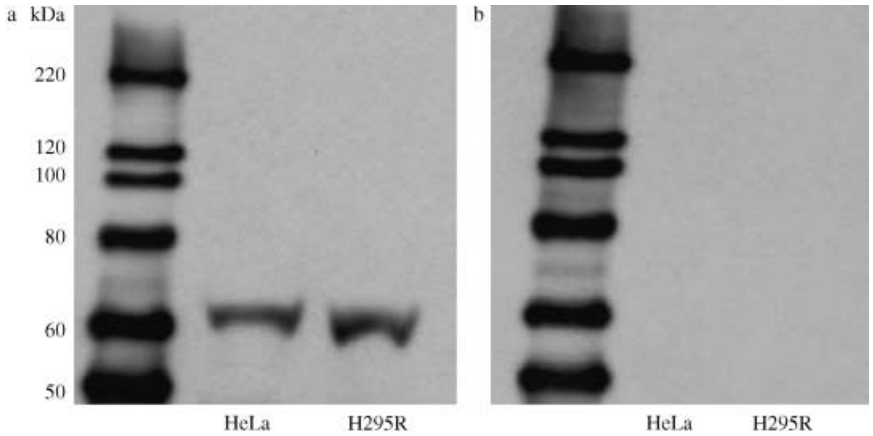

Fig. 1. Immunoblot analysis including immunoabsorption test. (a) Only a single band of 60 kDa was detected for DAX-1 in the HeLa and H295R control cell lines. Preparations were also incubated with DAX-1 monoclonal antibody preparations preabsorbed with synthetic antigen peptide as primary antibody. (b) No specific band was detected in these sections.

antibody (Sigma-Aldrich, Saint Louis, MO, USA) for 30 min at 37°C. The slides were incubated with streptavidin-alkaline phosphatase conjugate for 16 min at 37°C. The signal was subsequently detected automatically using a BlueMap NBT/BCIP substrate kit for 6 h at 37°C. The slides were counterstained with Fast Red for 5 min prior to mounting.

Statistical analysis

Statistical analysis was performed by using SAS software (StatView, Version 4.11, Cary NC, USA). The statistical significance of the relationship between DAX-1 immunoreactivity and other parameters (grade, stage, age, recurrence rate, ERγ, ERβ, PR, Ki67 immunoreactivity) were evaluated using the χ^2 test. Statistical differences between DAX-1 immunoreactivity for stromal cells and epithelial cells were evaluated using the χ^2 test. The statistical significance of the relationship between DAX-1 immunoreactivity and the amounts of aromatase mRNA in human endometrial carcinoma were also evaluated using the Mann–Whitney *U*-test. The statistical significance of the relationship between DAX-1 immunoreactivity in typical and atypical hyperplasia was evaluated by using the Mann–Whitney *U*-test. The statistical significance of the relationship between DAX-1 LI and DAX-1 mRNA levels was determined by using a correlation coefficient (*r*) and regression equation. The statistical significance of the differences between DAX-1 immunoreactivity for endometrial carcinoma, endometrial hyperplasia and normal endometrium during the proliferative and secretory phases was determined by using Scheffe's *F*-test. The overall and disease-free survival curves were generated according to the Kaplan–Meier method, and the differences in survival were evaluated by using a log-rank test. *P*-values less than 0.05 were considered to be significant.

Results

Immunoblot analysis

The results of the immunoblot analysis are summarized in Figure 1. Only a single band of 60 kDa was detected for DAX-1 in the HeLa and H295R control cell lines (Fig. 1a). The antibody preabsorbed with synthetic antigen peptide as a primary antibody demonstrated no specific band (Fig. 1b).

mRNA *in situ* hybridization

The results of the mRNA *in situ* hybridization study are summarized in Figure 2. Accumulation of mRNA hybridization signals for DAX-1 was detected predominantly in the cytoplasm of the adrenal cortex (Fig. 2a,b). Negative controls using the sense probe for DAX-1 mRNA yielded no significant accumulation of mRNA hybridization signals of this peptide (Fig. 2c). Patterns of accumulation of mRNA hybridization signals were consistent with the results of DAX-1 immunohistochemistry (Fig. 2d).

Normal cycling endometrium

DAX-1 immunoreactive protein was detected in the nuclei of both epithelial and stromal cells (Fig. 3a,b). The results are summarized in Figure 4. The DAX-1 LI values in the secretory phase were 59.5 ± 34.8 (epithelial cells) and 54.0 ± 31.1 (stromal cells). The DAX-1 LI values in the proliferative phase were 31.2 ± 36.1 (epithelial cells) and 25.5 ± 27.7 (stromal cells). The DAX-1 LI in the secretory phase was significantly higher than that in the proliferative phase, both for epithelial ($P = 0.0415$) and stromal cells ($P = 0.01$). Ad4BP/SF-1 was not detected in either epithelial or stromal cells, neither in the proliferative phase nor the secretory phase (Fig. 3e).

Endometrial hyperplasia

DAX-1 immunoreactive protein was detected in the nuclei of both epithelial and stromal cells (Fig. 3c). No significant correlation was detected between the DAX-1 LI in typical and atypical hyperplasia, either for epithelial cells ($P = 0.31$) or stromal cells ($P = 0.27$). The DAX-1 LI in hyperplasia (epithelial cells 60.2 ± 33.0; stromal cells 50.8 ± 30.5) was significantly higher than that in normal endometrium during the proliferative phase, both for epithelial cells ($P = 0.0093$) and stromal cells ($P = 0.0067$; Fig. 4). Ad4BP/SF-1 was not detected in any of the cases of endometrial hyperplasia examined.

Endometrial carcinoma

Immunohistochemistry DAX-1 immunoreactive protein was detected in the nuclei of both epithelial and stromal cells (Fig. 3d). The DAX-1 LI values in carcinoma were 26.7 ± 31.8 (epithelial cells) and 21.1 ± 26.5 (stromal cells). There

 doi: 10.1111/j.1349-7006.2005.00101.x

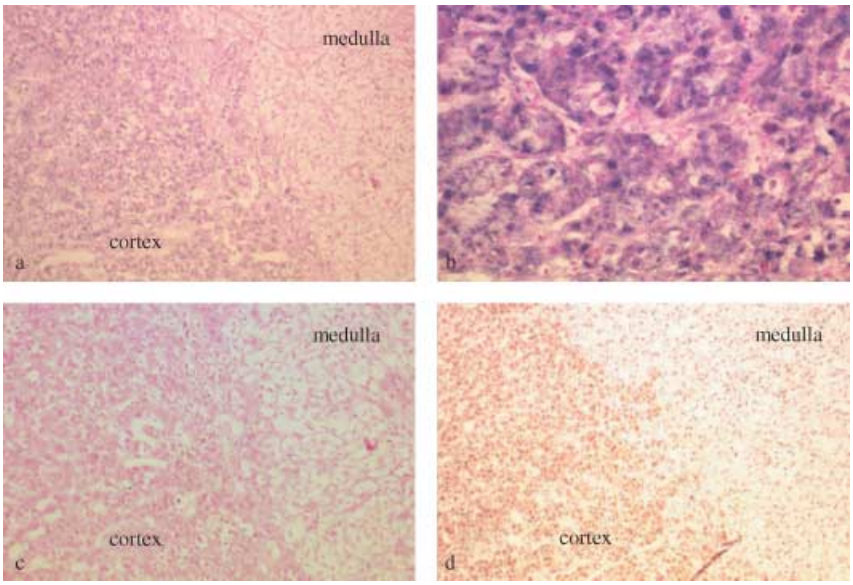

Fig. 2. mRNA *in situ* hybridization and immunohistochemistry for DAX-1 in adrenal gland tissue. (a,b) An accumulation of DAX-1 mRNA hybridization signals was detected predominantly in the cytoplasm of the cortex. (c) Negative controls using a sense probe for DAX-1 mRNA yielded no significant accumulation of mRNA hybridization signals of this peptide. (d) DAX-1 immunoreactivity was detected in the cortex. Original magnification ×100 (a,c,d), ×400 (b).

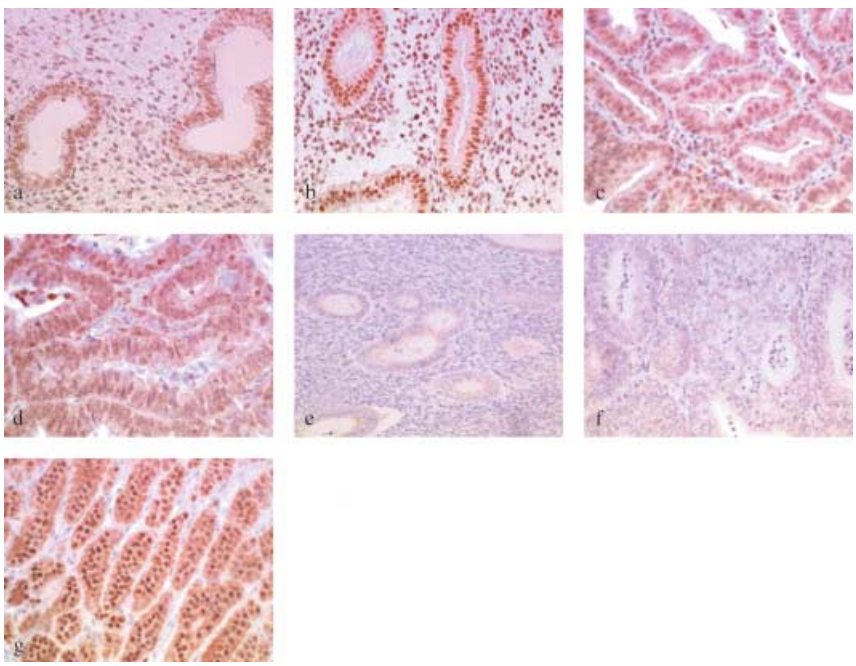

Fig. 3. Immunohistochemistry for DAX-1 and Ad4BP/SF-1 in normal endometria, endometrial hyperplasia and endometrial carcinoma. DAX-1 immunoreactivity was detected in the nuclei of both epithelial and stromal cells of normal endometria obtained during (a) the proliferative phase and (b) the secretory phase, and (c) endometrial hyperplasia, and (d) endometrial carcinoma. Ad4BP/SF-1 immunoreactivity was not detected in (e) normal endometria or (f) endometrial carcinoma. (g) Adrenal gland tissue was the positive control for the immunohistochemical studies. Original magnification × 400.

was a significantly positive correlation between the DAX-1 immunoreactivity in epithelial and stromal cells ($P < 0.0001$). There were no significant correlations between DAX-1 immunoreactivity and PR immunoreactivity, Ki67 immunoreactivity, stage, age, or recurrence rate of the patients examined (Tables 1,2). However, there was a significant inverse or negative correlation between DAX-1 immunoreactivity and histological grade of carcinoma, both for epithelial cells ($P = 0.0196$) and stromal cells ($P = 0.0003$; Table 1). In addition, a significant positive correlation was detected between DAX-1 and ERγ immunoreactivity ($P = 0.006$), and ERβ immunoreactivity ($P < 0.001$; Table 2). Comparisons among normal endometrium, hyperplasia and carcinoma are summarized in Figure 3. The DAX-1 LI in normal endometrium during the secretory phase was significantly higher than that in endometrial carcinoma, both for epithelial ($P = 0.001$) and stromal cells ($P < 0.0001$). The DAX-1 LI in endometrial hyperplasia was also significantly higher than that in endometrial carcinoma, also for both epithelial and stromal cells ($P < 0.0001$; Fig. 4). Ad4BP/SF-1 immunoreactivity was not detected in any of the cases of endometrial carcinoma examined in this study (Fig. 3f).

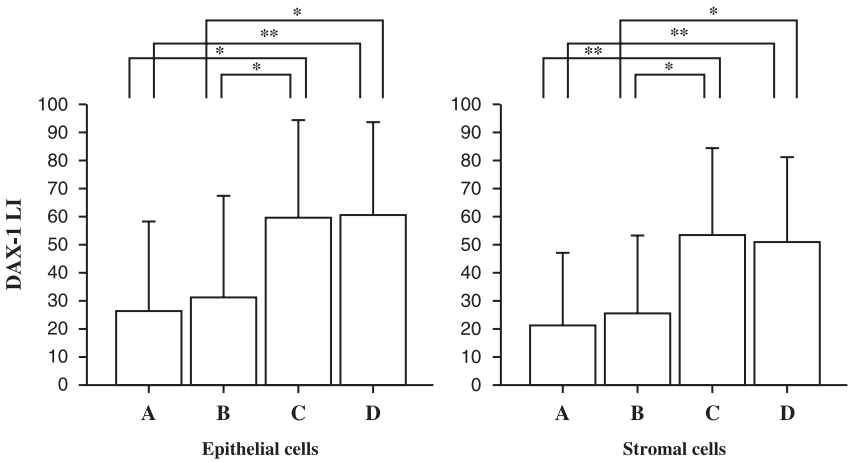

Fig. 4. DAX-1 labeling index (LI) in (a) endometrial carcinoma, (b) normal endometrium during the proliferative phase, (c) normal endometrium during the secretory phase, and (d) hyperplasia. The DAX-1 LI in normal endometrium during the secretory phase was significantly higher than that in endometrial carcinoma. Also, the DAX-1 LI in endometrial hyperplasia was significantly higher than that in endometrial carcinoma. The DAX-1 LI in the secretory phase was significantly higher than that in the proliferative phase. The DAX-1 LI in endometrial hyperplasia was significantly higher than that in normal endometrium during the proliferative phase. All values are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.0001$ (Scheffe's F-test).

Table 1. Correlation between DAX-1 immunoreactivity and other parameters in human endometrial carcinoma

	No. cases	DAX-1 expression in epithelial cells			DAX-1 expression in stromal cells		
		+	–	*P*-value	+	–	*P*-value
No. cases	103	46	57		44	59	
Grade							
G1, G2	81	41	40		42	39	
G3	22	5	17	0.0196	2	20	0.0003
Stage							
I a, b	49	23	26		18	31	
I c, II, III, IV	54	23	31	NS	26	28	NS
Age (years)							
≤ 50	21	8	13		8	13	
> 50	82	38	44	NS	36	46	NS
Recurrence							
+	16	7	9		6	10	
–	87	39	48	NS	38	49	NS

NS, not significant.

DAX-1 and Ad4BP/SF-1 mRNA expression in endometrial cancer tissues A significant positive correlation was detected between DAX-1 LI and amount of DAX-1 mRNA, in both epithelial cells ($r = 0.357$, $P = 0.0415$) and stromal cells ($r = 0.362$, $P = 0.0383$), by using both the correlation coefficient and regression equation. No significant correlations were detected between DAX-1 immunoreactivity and amounts of aromatase mRNA determined by quantitative RT-PCR (Table 2). Ad4BP/SF-1 mRNA expression was not detected in any of the cases of endometrial carcinoma examined in this study.

No significant correlations were detected between the status of DAX-1 and overall and disease-free survival of the patients.

Discussion

DAX-1 functions as a global negative regulator of steroid hormone production by repressing the expression of many genes involved in the steroidogenic pathway.[10] It has recently been demonstrated that aromatase mRNA levels in the testes, and circulating estrogen levels rise significantly in DAX-1-deficient mice, due to the lack of inhibition of Ad4BP/

Table 2. Correlation between labeling index (LI) for DAX-1 and other parameters in human endometrial carcinoma

	No. cases	DAX-1 expression		*P*-value
		+	–	
No. cases	103	46	57	
ERγ				
+	68	37	31	
–	35	9	26	0.006
ERβ				
+	50	35	15	
–	53	11	42	< 0.001
PR				
+	67	34	33	
–	36	12	24	NS
Ki67				
+	75	36	39	
–	28	10	18	NS
Aromatase (RT-PCR)	42	20.8 (0–21.7)* (n = 22)	22.1 (0–362.4)* (n = 20)	NS

*Data are presented as median (range). ER, estrogen receptor; PR, progesterone receptor; NS, not significant; RT–PCR, reverse transcription–polymerase chain reaction.

 doi: 10.1111/j.1349-7006.2005.00101.x

SF-1-mediated P450arom expression.[18] Gurates *et al*. also demonstrated that DAX-1 inhibits Ad4BP/SF-1-pathway-dependent P450arom expression in cultured human endometriotic glandular and endometrial stromal cells.[19] These *in vitro* studies clearly demonstrated the potentially pivotal roles of DAX-1 in the regulation of *in situ* steroidogenesis and steroid metabolism in human endometrium and its disorders, possibly through the inhibition of *in situ* steroid production. However, the status of DAX-1 in the human endometrium has remained virtually unknown. Therefore, we first determined the immunohistochemical localization of DAX-1 in endometrial carcinoma, endometrial hyperplasia and normal endometrium as a first step toward understanding the possible roles of DAX-1 in human endometrium.

DAX-1 immunoreactivity in endometrial carcinoma, endometrial hyperplasia and normal endometria was found in the nuclei of both epithelial and stromal cells. In human endometrium and in endometrial disorders, steroidogenesis is believed to occur in both epithelial and stromal cells.[37–40] There was a significant inverse correlation between DAX-1 immunoreactivity and histological grade in endometrial carcinoma. In addition, DAX-1 immunoreactivity in normal endometrium during the secretory phase and in endometrial hyperplasia was significantly higher than that in endometrial carcinoma. These results were consistent with the fact that aromatase was expressed at much higher levels in carcinoma than in normal endometrium and endometrial hyperplasia, and with the possible roles of DAX-1 in suppressing the expression of steroidogenic enzymes including aromatase, as has been demonstrated by the *in vitro* studies described earlier. The loss of expression or decreased expression of DAX-1 in endometrial carcinoma may therefore result in active or increased intratumoral steroid metabolism or production in endometrial carcinoma, which subsequently causes the estrogen-dependent proliferation of carcinoma cells. However, Ad4BP/SF-1 immunoreactivity was not detected in any of the cases of endometrial carcinoma or endometrial hyperplasia, or normal endometrium examined. Furthermore, no significant correlations were detected between DAX-1 immunoreactivity and amounts of aromatase mRNA determined by RT-PCR. Therefore, DAX-1 is by no means believed to regulate aromatase expression, which is one of the most important steps in *in situ* estrogen synthesis, through interaction with Ad4BP/SF-1 as proposed by *in vitro* studies on human endometrial carcinoma. This finding also suggests the existence of a transcriptional factor other than Ad4BP/SF-1. For example, liver receptor homolog 1 (LRH-1), which was reported to act in a fashion similar to Ad4BP/SF-1,[41,42] may be associated with the regulation of expression of intratumoral aromatase in human endometrial carcinoma as in breast cancer. However, further investigations are required for clarification.

DAX-1 has been reported by Zhang *et al*. to play important roles in the regulation of ER transactivation.[21] They also demonstrated that DAX-1 directly binds to ERγ and ERβ via the N-terminal repeat domain that contains L*XX*LL motifs. DAX-1 has been reported to inhibit the transcriptional activity of liganded ER by a sequential mechanism, possibly involving the recruitment of corepressors.[16] Accordingly, DAX-1 itself acts as an ER corepressor. In this study, there were significant positive correlations between DAX-1 immunoreactivity and ERγ LI or ERβ LI. The DAX-1 LI in normal endometrium during the secretory phase was significantly higher than that in the proliferative phase. The serum E2 level is known to reach a plateau, and ER expression persists in glandular cells during the secretory phase.[43] It is well known that less estrogenic actions are required in the endometrium following ovulation. These results all suggest that DAX-1 is likely to play a very important role in the maintenance of hormonal environments to prepare for implantation by acting as corepressor for ER, resulting in diminished local estrogenic actions together with 17β-hydroxysteroid dehydrogenase (17β HSD) type 2, which catalyzes the oxidation of 17β-estradiol (E2) to estrone (E1) in endometrial mucosa of the secretory phase.[40] Estrogens are also considered to play very important roles in the development of endometrial hyperplasia. Some cases of endometrial hyperplasia, especially atypical hyperplasia, are considered to be precursors of endometrial endometrioid adenocarcinoma.[44,45] In our present study, the DAX-1 LI in hyperplasia was significantly higher than that in normal endometrium during the proliferative phase or in endometrial carcinoma. Endometrial hyperplasia was in general believed to be caused by continuous estrogenic stimulation such as ovulatory dysfunction, which results in abnormal cell proliferation with altered patterns of expression for several genes.[46,47] The presence of DAX-1 in endometrial hyperplasia may therefore represent an *in situ* defensive mechanism for modulating unopposed estrogenic effects by acting as a corepressor for ER as proposed in human prostatic lesions.[25,26,48] There was a significant inverse correlation between DAX-1 immunoreactivity and histological grade of carcinoma. These findings also suggest that DAX-1 may inhibit cell proliferation and the progression of endometrial carcinoma in a manner similar to endometrial hyperplasia described earlier. DAX-1 may therefore provide a novel model of endocrine therapy of endometrial endometrioid adenocarcinoma as a selective estrogen receptor modulator (SERM). However, further *in vitro* investigations are required to clarify the precise roles of DAX-1 in regulating estrogenic activity in human endometrial carcinoma.

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 doi: 10.1111/j.1349-7006.2005.00101.x