and separated on a formaldehyde-3-[*N*-morpholino]propanesulfonic acid agarose gel and transferred to Hybond nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out in 5× standard saline citrate (SSC), 1% (wt/vol) sodium dodecyl sulfate, 5× Denhardt's solution, 100 μg/ml herring sperm DNA, and [α-^{32}P]deoxy-CTP-labeled DNA probe at 45 C overnight. The membrane was washed in 2× SSC/0.1% (wt/vol) sodium dodecyl sulfate for 30 min, followed by intensive washing in 1× SSC/0.1% (wt/vol) sodium dodecyl sulfate at 45 C for 30 min with two changes of the washing solution. The membrane was exposed to an imaging plate and analyzed using an image analyzer (BAS-2000, Fuji Photo Film Co., Ltd., Tokyo, Japan). For RT-PCR, 4 μg total RNA were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(deoxythymidine) [oligo(dT)] primer. The following primer sets were used for PCR: DDV10-1, 5′-TCGGTTTGACAACGAGAAGG-3′; and DDV10-2, 5′-CTAGACAGGAACAGGAG-TTG-3′ for DDV10; DDV10-3, 5′-TAGGTTTGACAACCAGGATG-3′; and DDV10-4, 5′-CTAGGAAGGAAAAAAAGGAG-3′ for OCIL; and GAP-1, 5′-ATGAC-CACAGTCCATGCCATCAC-3′; and GAP-2, 5′-TCATTGTCATAC-CAGGAAATGAG-3′ for glyceraldehyde phosphate dehydrogenase (GAPDH). Twenty-eight cycles of amplification were carried out under the following conditions: denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec, and extension at 72 C for 1 min. At completion of the PCR, fragments were resolved on 1.5% agarose gels. First strand cDNA was synthesized from 2 μg total RNA for real-time Q-PCR. The following specific primers were used: DDV10-5, 5′-TCTAAGCACCCTTGGAT-GTGGA-3′; and DDV10-6, 5′-AGCTTTCGCCTTCTTCTGGCT-3′ for DDV10; and GAP-3, 5′-AACGACCCCTTCATTGACCTC-3′; and GAP-4, 5′-CCTTGACTGTGCCGTTGAATT-3′ for GAPDH. PCR was carried out using ABI PRISM 5700 (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, except that 40% of the suggested reaction volume (20 μl) was used. PCR conditions were 2 min at 50 C, 95 C for 10 min, and 40 cycles at 95 C for 15 sec and 60 C for 1 min. Gene expression levels were normalized to GAPDH mRNA expression. To establish standard curves for each gene, serial dilutions of a known amount of cDNA sample were used. The C_T (threshold cycle) values for each gene were plotted on these standard curves to obtain the amount of copies present in the initial cDNA sample. All samples were run in triplicate and replicated in three independent experiments, and the mean C_T for GAPDH was subtracted from the DDV10 mean C_T for normalization.

Experimental animals

C57BL/6 mice and ERα knockout (αERKO) mice (9) were maintained on a commercial diet (CE-1, CLEA, Tokyo, Japan) and tap water and were kept at 24 C under 12-h light, 12-h dark cycle. Female mice were given five daily sc injections of 3 μg diethylstilbestrol (DES; Sigma-Aldrich Corp., St. Louis, MO) dissolved in sesame oil or of the oil vehicle alone starting on the day of birth. Mice were ovariectomized at 7–8 wk of age, and tissues were dissected 10 d later. Tissues were flash-frozen in liquid nitrogen and stored at −80 C until RNA extraction or fixed for histology. For the study of estrogenic effects, E2 (1.0 or 0.25 μg/animal; Sigma-Aldrich Corp.), progesterone (1.0 μg/animal; Sigma-Aldrich Corp.), or the ER antagonist ICI 182,780 (50.0 μg/animal; TOCRIS, Ellisville, MO) were injected sc into ovariectomized mice. All experiments and animal husbandry protocols were approved by the animal care committee of Okazaki National Research Institutes.

Tissue separation

Tissue separation procedures for vaginal epithelium and stroma have been previously described (10). Briefly, vaginas from mice were trimmed, opened lengthwise, and incubated with 0.5% trypsin (Invitrogen, Carlsbad, CA) in calcium- and magnesium-free Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) for 90 min at 4 C. Vaginal stroma and epithelium were then separated by removing the epithelium from the underlying stroma using a fine forceps.

Differential display/RT-PCR (DD/RT-PCR) and sequencing of DD/RT-PCR fragments

Total RNA (2 μg) isolated from vagina was reverse transcribed using rhodamine-labeled oligo(dT) primer (5′-rhodamine labeled-T_nGC). The resulting cDNAs were amplified by PCR using rhodamine-labeled oligo(dT) downstream primer and upstream arbitrary primers (AP-1, 5′-GATCATGGTC-3′). Samples from each amplification reaction were loaded onto a 5% nondenaturing polyacrylamide gel and electrophoresed at 30 mA for 3 h. The gel image was analyzed to detect differentially expressed, rhodamine-labeled DNA fragments using a Fluoro-Image Analyzer (FLA-3000G, Fuji Photo Film Co., Ltd.). Bands of interest were excised from the gel, and the cDNA was eluted and reamplified by PCR using the same primers as those used in the original PCR amplification. The amplified DNA fragment was recovered from the gel and subcloned into pGEM-T Easy vector using the T/A cloning system (Promega Corp., Madison, WI). The clones were sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems) with T7 primer and analyzed on the ABI PRISM 377 automatic sequencer (PE Applied Biosystems).

Plasmids

The full-length DDV10 and OCIL cDNAs were isolated from mouse vaginal RNA using a PCR-based technique and subcloned with T/A subcloned as described above. Amplification was performed for 30 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min using the 5′ primer DDV10-7 (5′-TCTGAGATGAACATTACAAGG-3′) and the 3′ primer DDV10-2 for DDV10, and the 5′ primer DDV10-8 (5′-ATGTGT-GTCACAAAGGCTTC-3′) and the 3′ primer DDV10-4 (for OCIL). All clones were sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems) with T7 and SP6 primers and analyzed on the ABI PRISM 377 automatic sequencer (PE Applied Biosystems).

Histological observation

For immunocytochemistry, vaginas were fixed in 10% formaldehyde neutral buffer solution (Nacalai, Kyoto, Japan) embedded in paraffin, serially sectioned at 6 μm, deparaffinized, rehydrated, and washed twice in PBS for 10 min each time. Sections were then stained with Histofine (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Antikeratin polyclonal antibody (Nichirei) and antimouse keratin 1 antibody (BabCo, Richmond, CA) were used as primary antibodies. The expression of DDV10 mRNA was examined by *in situ* hybridization using digoxygenin-UTP-labeled probes translated *in vitro* the from DDV10 clone. We selected the cytoplasmic domain of DDV10 as a probe to distinguish between DDV10 and OCIL. Vaginas were fixed with 4% paraformaldehyde in PBS for 16 h at 4 C. Preparation of the digoxygenin-labeled probe, conditions of hybridization and washing, and detection of the signal were performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN).

Data analysis

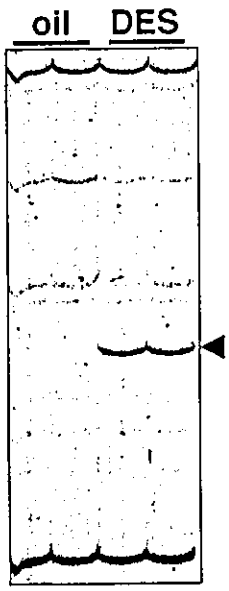
Statistical analysis was carried out using *t* test or Welch's test. All data are reported as the mean ± SEM. In all cases, means were considered significantly different at $P < 0.05$.

Results

Identification of a novel C-type lectin by differential display/RT-PCR

The proliferation and cornification of vaginal epithelium are regulated by estrogen; however, the molecular mechanisms underlying estrogen-dependent cell proliferation and differentiation of vaginal epithelium are unclear. Neonatal DES treatment causes estrogen-independent cell proliferation and differentiation (11–15). Therefore, the vaginas of DES-treated mice provide a good system for analyzing the proliferation and cornification of vaginal epithelium. We compared the transcripts from ovariectomized (OVX) mouse vaginas treated neonatally with either oil vehicle (oil-OVX-vagina) or DES (DES-OVX-vagina) using DD/RT-PCR. A differentially expressed DNA fragment was detected in samples from DES-OVX mice (Fig. 1A, designated DDV10) and

A



B

GATCATGGTCCTCACTGTAGCTGTAGTTGCTCTTTCTGTT
GCTTTGCCAGCAACAAAGACAGAACAGATCCTAATCAACA
AGACCTATGCTGCTTGCCCGAAAAACTGGATTGGAGTTGG
AAATAAATGTTTTTATTTTTCTGAATACACAAGTAACTGG
ACATTCGCCCAGACCTTCTGCATGGTACAAGAGGCCCAAC
TAGCTCGGTTTGACAACGAGAAGGAGCTGAATTTCCTAAT
GAGATACAAGGCAAATTTTGATTCCTGGATTGGACTGCAC
AGAGAGTCGTCAGAGCACCCTTGGAAGTGGACAGACAACA
CTGAGTATAACAACATGATTCCCATCCAGGGAGTGGAAAC
ATGTGCCTACCTGAGCGGCAATGGGATCAGCAGTTCCAGG
CACTATATACCTCGGATATGGATCTGTAGCAAGCTTAACA
ACTATAGCCTCCACTGCCCAACTCCTGTTCCTGTCTAGCA
TTTACCAAGAGACTCTTCCTAGCCTGTTATCTATGGGTGC
TACTTTTTCCCCTATGGTCCCACAGTGCTATCAAACGGGA
TTGAGAATATTTTTTAACGTC GCAAAAAAAAAAAAAAA

FIG. 1. Identification of DES-induced mRNA by DD/RT-PCR. A, Mice were neonatally treated with oil or DES for the first 5 d after birth. Mice were OVX 7 d after birth, and vaginas were dissected from animals 10 d later. Total RNA was isolated, and DD/RT-PCR/gel analysis was performed. The *arrow* indicates the band that is differentially expressed in the vaginas of neonatally DES-treated mice. B, Nucleotide sequence of DDV10. Two *underlined* sequences were used as primers for DD/RT-PCR as upstream and oligo(dT) primer, respectively.

was isolated from the gel, reamplified, subcloned, and sequenced. This cDNA fragment is 598 bp long (Fig. 1B; DDBJ/EMBL/GenBank accession no. AB091386). BLAST analysis of this partial cDNA against the nonredundant GenBank/EMBL database revealed that the DDV10 clone is identical with a cDNA (4632413B12 gene) of unknown function that was isolated by the RIKEN cDNA project (16). DDV10 encodes a 269-amino-acid putative membrane protein with structural homology to the C-type lectin family. The predicted DDV10 protein has a 142-amino-acid extracellular domain, a 21-amino-acid transmembrane domain, and a 106-amino-acid cytoplasmic domain. The extracellular domain contains five cysteine residues and three predicted *N*-linked glycosylation sites at residues 137, 163, and 221. A myristoylation motif is also predicted in the intracellular domain. As shown in Fig. 2, the amino acid sequence of DDV10 is similar to that of mouse OCIL (osteoclast inhibitory lectin) that was isolated as an inhibitor of osteoclast formation (17) (81% identical in extracellular domain, 60% overall). As DDV10 was identified as a differentially expressed band by DD/RT-PCR, we confirmed its expression by RT-PCR using gene-specific primer sets. As expected, the expression level of DDV10 was high in DES-OVX-vagina. In contrast to DDV10 expression, OCIL was high in oil-OVX-vagina in which the epithelium is composed of two or three layers of cuboidal cells (Fig. 3).

Expression of DDV10 and OCIL mRNAs in vagina

To analyze the effect of estrogen on DDV10 expression, oil vehicle, E2, or E2 plus the ER antagonist ICI 182,780 were injected into mice 10 d after ovariectomy (OVX). Thirty hours after injection, vaginas were dissected, total RNA was isolated, and potentially contaminating genomic DNA was removed by deoxyribonuclease treatment, cDNAs were synthesized and used for Q-PCR. The steady-state levels of DDV10 transcripts were increased by E2 treatment, and this increase was blocked when E2 was accompanied by ICI 182,780 (Fig. 4A). We found that, like E2, DES can induce the expression of DDV10 (data not shown), whereas progesterone treatment does not (Fig. 4B). To more precisely analyze estrogenic effects on DDV10 expression, mice were OVX, and vaginas were dissected at 24-h intervals after OVX. Transcript levels were examined by Northern blot or Q-PCR. DDV10 transcripts were decreased by OVX; however, OCIL mRNA increased slightly (Fig. 5, A and B). E2 administration 10 d after OVX led to an increase in the levels of DDV10 transcripts approximately 12 h after treatment (Fig. 5C), whereas E2 treatment slightly decreased OCIL mRNA levels (Fig. 5D).

Tissue distribution of DDV10 and OCIL mRNAs

To determine the distribution of DDV10 and OCIL mRNAs, total RNA was isolated from various tissues from male and female mice, and RT-PCR was performed using gene-specific primers. Intriguingly, OCIL was expressed in both vagina and uterus, whereas DDV10 was expressed only in vagina (Fig. 6A). We could not detect DDV10 in the uterus of E2-treated mice (data not shown). OCIL mRNA was detected in all tissues examined as described by Zhou *et al.* (17) (Fig. 6B). DDV10 mRNA was detected in eye, tongue, stomach, and spleen (Fig. 6B). These results suggested that DDV10 may be involved in the stratification and/or cornification of the epithelium. However, the spleen does not have a stratified and cornified epithelium, and the function of DDV10 in spleen will probably be different from that in epithelia.

```
DDV10: MNITRASLPMLNTTCSCRREKWNFLGRYEGTFDYWIGLHR
OCIL : MCVTKASLPMLSPTGS----------------------
                                                  ◄
DDV10: ASSKHPWMWTDNTEYNNMFVYHMNAQCLKKPEEGESSPGT
OCIL : ----------------------------PQEVEVG---
                                    Intracellular Domain
DDV10: GGVHSYKILQRNSLRAISPESSAKLYCCCGVIMVLTVAVV
OCIL : ------KILQGKRHGTISPESCAKLYCYYGVIMVLTVAVI
                                    ◄──────────►
                                    Transmembrane Domain
DDV10: ALSVALPATKTEQILINKTYAACPKNWIGVGNKCFYFSEY
OCIL : ALSVALSATKTEQIPVNKTYAACPQNWIGVENKCFYFSEY
       ◄──────►◄──────────────────────────────►
              Neck Domain
DDV10: TSNWTFAQTFCMAQEAQLARFDNEKELNFLMRYKANFDSW
OCIL : PSNWTFAQAFCMAQEAQLARFDNQDELNFLMRYKANFDSW
                                    C-lectin Domain
DDV10: IGLHRESSEHPWKWTDNTEYNNMIPIQGVETCAYLSGNGI
OCIL : IGLHRESSEHPWKWTDNTEYNNTIPIRGEERFAYLNNNGI
DDV10: SSSRHYIPRIWICSKLNNYSLHCPTPV-PV
OCIL : SSTRIYSLVMWICSKLNSYSLHCQTPFFPS
                                    ◄──────────►
                                    Tail
```

FIG. 2. Sequence alignment of DDV10 and OCIL. Deduced amino acid sequences of DDV10 (RIKEN cDNA 4632413B12, GenBank accession no. AK014570) and mouse OCIL (accession no. AF 321553) are shown. The cytoplasmic, transmembrane, and extracellular domains are indicated. Identical residues are marked with an *asterisk*.

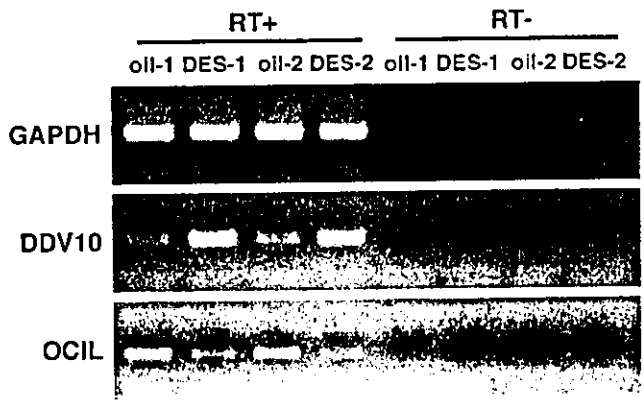


FIG. 3. Verification of the gene expression of the transcript identified by DD/RT-PCR. Total RNA was isolated from vaginas of neonatally DES-treated and oil-treated mice. RT-PCR was used to determine the expression levels of DDV10, mouse OCIL, and GAPDH mRNAs. As a control, RT (−) reaction mixture was used.

DDV10 expression during postnatal ontogeny

The vaginal epithelium of newborn mice is a pseudostratified epithelium composed of two or three layers, and this state continues to change about 20 d after birth (Fig. 7A). Keratins constitute a family of approximately 20 related α-helix-rich structural proteins of 40–70 kDa that are subunits of the intermediate filaments present in all epithelial cells (18). Keratin 1 is the predominant cytoskeletal intermediate filament protein expressed in epithelial cells during the transition from the proliferative to the terminal differentiation stage. Antikeratin 1 antibody-positive cells in vaginal epithelium were not found 5 and 20 d after birth (Fig. 7A). The production of E2 begins approximately 20 d after birth, and the vaginal epithelium proliferates and differentiates in response to increased E2 levels. Accordingly, antikeratin 1 antibody-positive cells are detected in the epithelium by 30 d after birth (Fig. 7A). Almost all vaginal epithelial cells were recognized by antikeratin 1 antibody (Fig. 7A). Q-PCR analysis did not detect DDV10 mRNA 20 d after birth, but by 30 d of age, the vaginal epithelium was multilayered, and a high level of DDV10 expression was observed (Fig. 7B).

DDV10 is not expressed in vaginas of αERKO mice

Estrogens promote vaginal epithelial proliferation and differentiation (19). These events require ERα, as shown by the lack of vaginal epithelial proliferation, stratification, and cornification in E2-treated αERKO mice (20). Antikeratin antibody recognized the vaginal epithelium of αERKO mice. However, antikeratin 1-positive staining was not detected in epithelial cells (Fig. 8A). RNAs from vaginas and tongues of wild-type and αERKO mice were analyzed for the expression of DDV10 mRNA by RT-PCR. As shown in Fig. 6B, DDV10

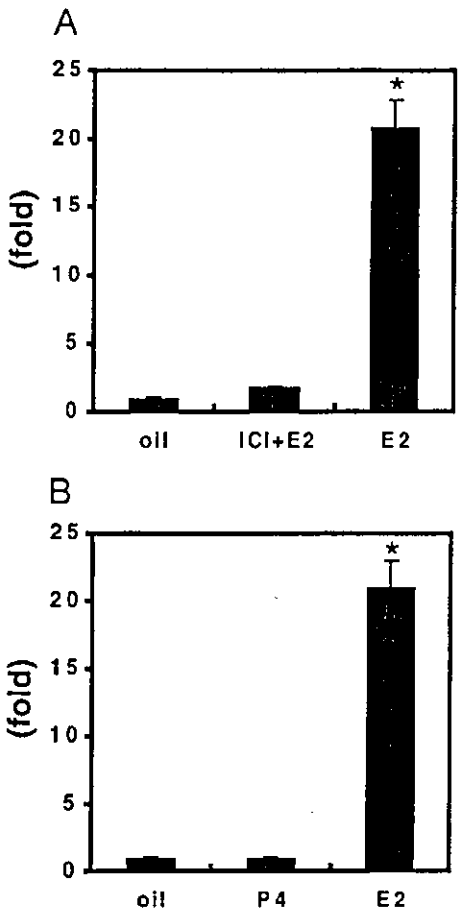


FIG. 4. Induction of DDV10 expression by E2. A, Expression level of DDV10 from vaginas of OVX mice after vehicle treatment (lane 1, oil), ICI plus E2 (lane 2, 50 μg ICI and 0.25 μg E2/animal), and E2 (lane 3, 0.25 μg E2/animal). *, $P < 0.01$ *vs.* oil and ICI plus E2. B, Expression level of DDV10 from vaginas of OVX mice after vehicle (lane 1, oil), progesterone (P4; lane 2, 1.0 μg P4/animal), and E2 (lane 3, 1.0 μg E2/animal). Results are the mean ± SEM of three experiments, each performed in triplicate. *, $P < 0.01$ *vs.* oil and P4.

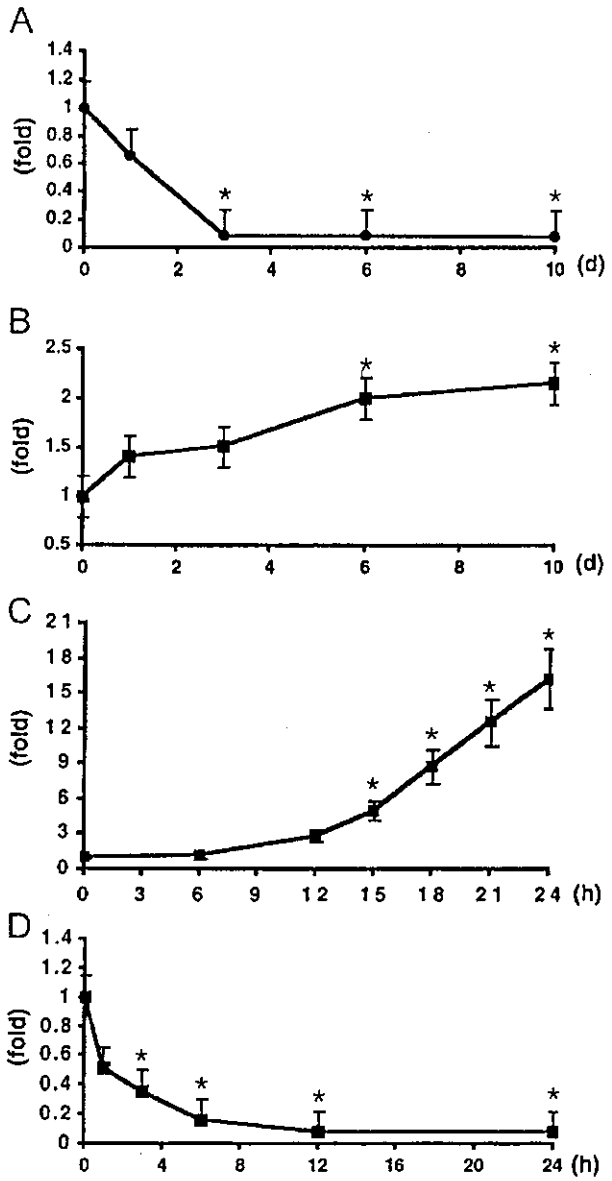


FIG. 5. Regulation of DDV10 and OCIL mRNA expressions by E2. Mice were OVX at 50 d of age (designated d 0), and vaginas were dissected from animals 0, 1, 3, 6, and 10 d later (A and B). Total RNA was isolated from vaginas, and Q-PCR analysis (A for DDV10 mRNA) and Northern blot analysis (B for mOCIL mRNA) were carried out. Fifty-day-old mice were OVX, and a single injection of E2 (1 μg/animal) was given 10 d later. Vaginas were dissected from animals 0, 6, 12, 15, 18, 21, and 24 h after the injection (C) and 0, 1, 3, 6, 12, and 24 h after the injection (D). Total RNA was isolated from vaginas, and mRNA levels were analyzed by Q-PCR (C for DDV10 mRNA) or Northern blot (D for mOCIL mRNA). Results are the mean ± SEM of three experiments, each performed in triplicate. *, $P < 0.05$ *vs.* before treatment.

mRNA was expressed in both vagina and tongue of wild-type mice. In αERKO mice, DDV10 mRNA was detected in tongue, but not in vagina (Fig. 8B). E2 injection into αERKO mice was unable to elicit detectable expression of DDV10 in vagina (data not shown).

DDV10 mRNA is expressed in vaginal epithelial cells, but not stromal cells

Our data suggested that DDV10 may be involved with the proliferation, stratification, and cornification of vaginal epithelium; therefore, we next examined whether DDV10 mRNA is expressed in epithelium or stroma. The vaginal epithelium was separated from the stroma, and RNA was prepared from each tissue and analyzed by Q-PCR. We found that DDV10 mRNA was present in epithelium, but not in stroma (data not shown). We confirmed this localization using *in situ* hybridization. To distinguish DDV10 from OCIL, an RNA probe complementary to amino acids 1–98 of DDV10, was used. As expected, DDV10 mRNA was detected in epithelium, but not in stroma (Fig. 9). Furthermore, DDV10 was localized in suprabasal cells, but not in basal cells that proliferate in response to estrogen. These results suggest that DDV10

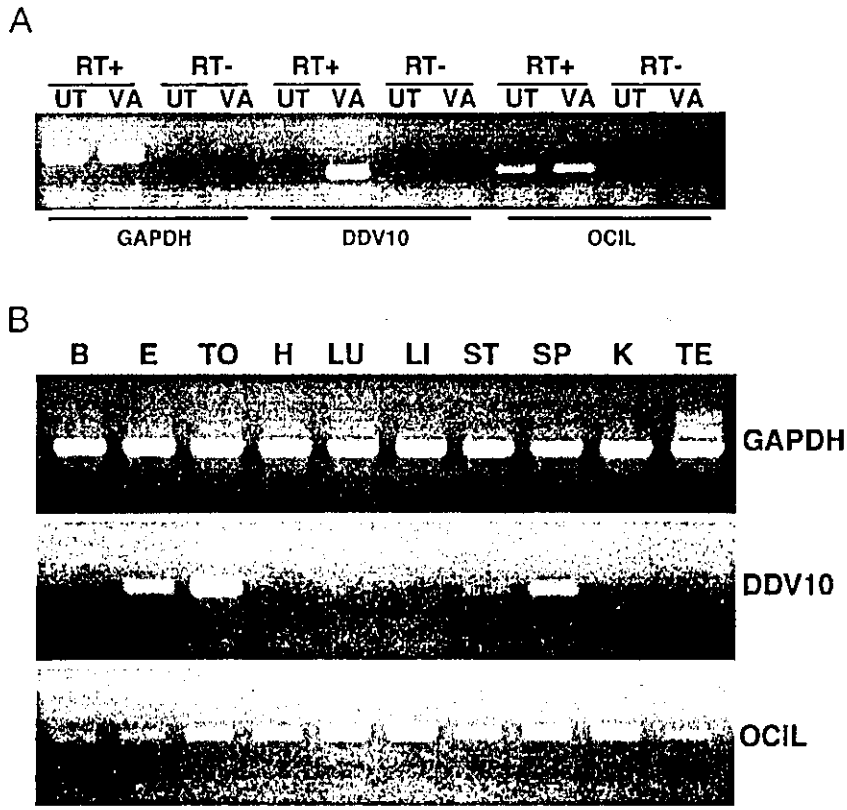


FIG. 6. Tissue distribution of DDV10 and OCIL mRNA. A, Total RNA was isolated from uteri (UT) and vaginas (VA). cDNA was synthesized with (RT+) or without (RT−) reverse transcriptase, and expressions of DDV10, mouse OCIL, and GAPDH mRNAs were analyzed by RT-PCR. B, Total RNA was prepared from brain (B), eye (E), tongue (TO), heart (H), lung (LU), liver (LI), stomach (ST), spleen (SP), kidney (K), and testis (TE). GAPDH was used as a positive control. mRNAs of DDV10 and mOCIL were detected by RT-PCR.

may be involved in stratification and/or cornification, but not proliferation, of vaginal epithelial cells.

Discussion

Estrogens regulate the proliferation and differentiation of cells in the mouse vagina. However, the molecular mechanism underlying estrogen-induced cell proliferation and differentiation have not been defined. In this study we reported the molecular cloning and characterization of a putative C-type lectin, termed DDV10, which may be involved in the stratification and/or cornification of vaginal epithelium. The predicted structure suggests that DDV10 encodes a type II transmembrane protein containing a carboxyl-terminal extracellular domain and an amino-terminal cytoplasmic domain. We showed that DDV10 is expressed in stratified, cornified epithelia of eye, tongue, stomach, and epithelial cells, but not stromal cells, in vagina. DDV10 is not expressed in vagina until 20 d after birth; moreover, vaginal expression is drastically decreased after OVX, but returns approximately 12 h after E2 treatment. DDV10 mRNA is undetectable in vaginas of ERαKO mice. We infer from these results that DDV10 is a novel, E2-regulated, C-type lectin.

Lectins are nonenzymatic sugar-binding proteins that bind with considerable specificity to complex carbohydrates found on secreted glycoproteins, cell surface glycoproteins, and extracellular matrix proteins. Lectins are involved in numerous cellular processes, such as host-pathogen interactions, targeting of proteins within cells, and cell-cell interactions (21, 22). The calcium-dependent (C-type) lectin family consists of two subtypes: soluble C-type lectin and transmembrane C-type lectin. C-Type lectins, include cell adhesion molecules such as selectins, which target leukocytes to lymphoid tissues and sites of inflammation (23, 24); mannose-binding proteins, which function in antibody-independent host defense against pathogens (25, 26); and leticans, a family of chondroitin sulfate proteoglycans including aggrecan, versican, neurocan, and brevican (27, 28). Each of these proteins contains a C-type carbohydrate recognition domain attached to other domains responsible for the physiological functions of the molecule (29). A new C-type lectin related to DDV10 called OCIL (osteoclast inhibitory lectin) was recently cloned and characterized (17). DDV10 has a high homology to mouse OCIL. The C-type lectin domains of DDV10 and OCIL amino acid sequences are 83% identical in the C-type lectin domain, but only 32% identical in the cytoplasmic domain. No further similarity to other proteins or putative conserved domains has been detected. We note that the cytoplasmic domain of DDV10 consists of 106 amino acids (Fig. 2); therefore, DDV10 may interact with another protein(s) through its cytoplasmic domain and could be involved in the regulation of calcium-dependent signal transduction. We compared the expression of DDV10 and OCIL in vagina and other tissues and found that DDV10 is expressed in proliferated vaginal epithelium. DDV10 mRNA levels rapidly decrease, whereas the expression of OCIL gradually increased after OVX. E2 treatment induced DDV10 expression and repressed OCIL expression. DDV10 mRNA levels change during the normal reproduc-

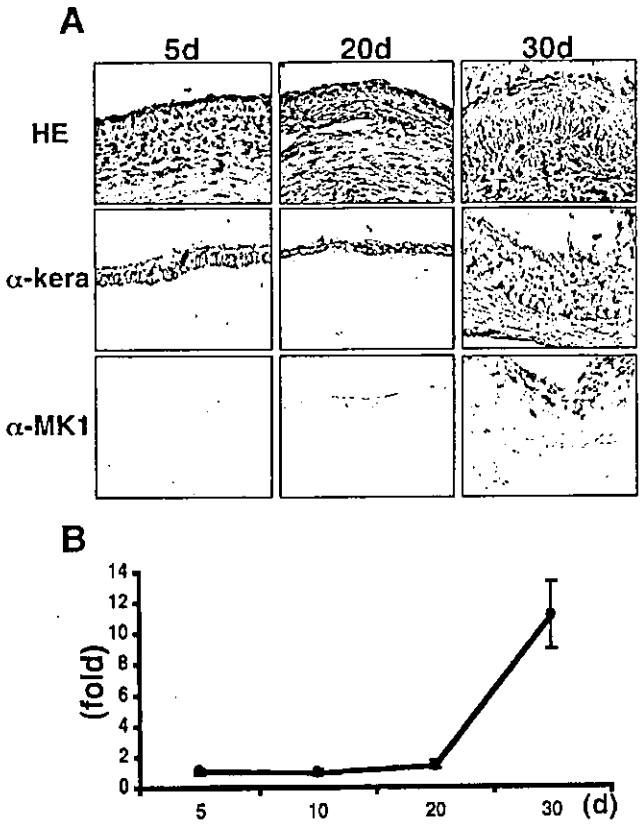


FIG. 7. Expression of DDV10 during postnatal ontogeny. A, Histological changes in vaginal epithelium. Vaginas were dissected from animals 5, 20, and 30 d after birth. Vaginas were fixed and stained with hematoxylin-eosin or immunostained with antikeratin antibody (α-kera) and antimouse keratin 1 antibody (α-MK1). Magnification, ×200. B, Total RNA was isolated from vagina 5, 10, 20, and 30 d after birth, and the DDV10 mRNA level was analyzed by Q-PCR. Results are the mean ± SEM of three experiments, each performed in triplicate. *, $P < 0.05$ *vs.* 5 d.

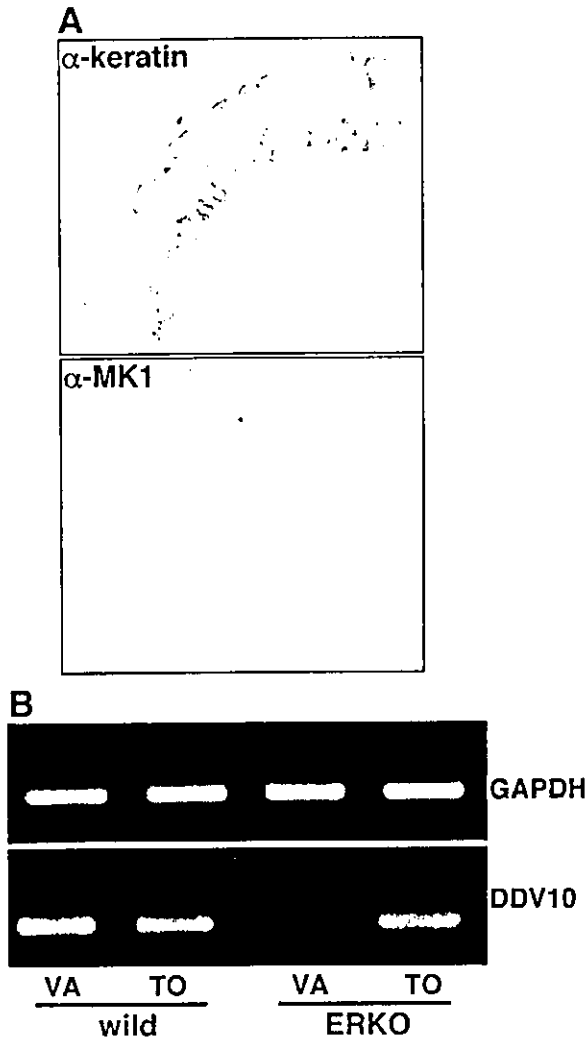


FIG. 8. DDV10 is not expressed in vaginas of αERKO mice. A, Immunohistochemistry was carried out on a section of vagina from αERKO mice. Antikeratin antibody (α-keratin; *upper panel*) and antimouse keratin 1 antibody (α-MK1; *lower panel*) were used. Magnification, ×200. B, Total RNA was prepared from vagina (V) and tongue (T) of wild-type and αERKO mice, and the cDNA was synthesized. The expression of DDV10 and GAPDH mRNAs was analyzed by RT-PCR.

tive cycle and are high at estrus (data not shown). Taken together, these results suggest that estrogens may positively regulate DDV10 while negatively regulating expression of OCIL. It is currently unknown whether the effects of estrogens on the expression of DDV10 are direct or indirect. Unlike c-Jun and c-Fos, which rapidly increase after E2 treatment (30–32), DDV10 expression was not detectable until 12 h after E2 treatment in OVX mice. The putative DDV10 promoter does not contain a consensus E2 response element (data not shown), and the expression of DDV10 in stomach appears to be E2 independent (data not shown). Therefore, it is likely that DDV10 is indirectly regulated by estrogens, but further study is required to establish this point.

Vaginal expression of DDV10 mRNA was rarely detected 20 d after birth, but was routinely detected 30 d after birth. We found that the expression of DDV10 began approximately 20 d after birth, and that DDV10 expression increased as the epithelial cells proliferated in response to increasing endogenous E2 levels (data not shown). In agreement with this model, DDV10 mRNA was not detected in vaginas of αERKO mice, which have two or three layers of atrophied, noncornified epithelium. *In situ* hybridization revealed that DDV10 was expressed in vaginal epithelial cells of the suprabasal cell layers, but not the proliferating basal cell layer, suggesting that it functions during epithelial differentiation, but not proliferation. We previously showed that neuropsin/kallikrein 8 is expressed in the mouse vagina and could not be detected until 24 h after E2 treatment (33) in contrast with DDV10. Thus, like neuropsin/kallikrein 8, DDV10 is a marker of epithelial differentiation and may act as a differentiation-regulating factor, perhaps playing a role in the initial events during the differentiation of vaginal epithelium.

Calcium is an important regulator of keratinocyte differentiation (7). Incubation of cultured keratinocytes with cal-

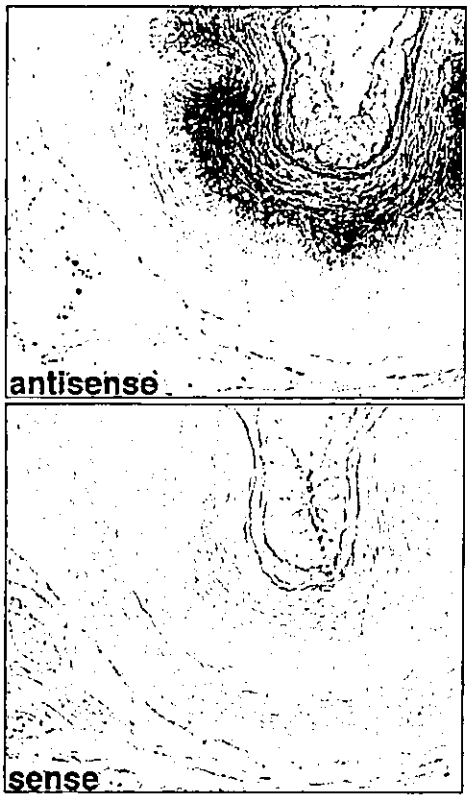


FIG. 9. Expression of DDV10 in vaginal epithelial cells. *In situ* hybridization was carried out on a section of vagina at the stage of estrus. DDV10 mRNA expression was observed in suprabasal cells in vaginal epithelium (*upper panel*, antisense probe; *lower panel*, sense probe). Magnification, ×100.

cium increases differentiation and the expression of differentiation-associated genes (34–36). Within hours of calcium treatment, keratinocytes shift from production of the basal keratins K5 and K14 to keratins K1 and K10, followed, subsequently, by increased levels of profilaggrin (the precursor of filaggrin, an intermediate filament-associated protein), involucrin and loricrin (precursors for the cornified envelope) (37, 38). An *in vivo* epithelial gradient of calcium has been identified with increasing calcium levels in the more differentiated layers. This suggests that calcium plays an important role in regulating epidermal differentiation (39). However, the mechanism by which the increase in extracellular free calcium triggers differentiation is not well understood. One possible mechanism involves the calcium-dependent activation of protein kinase C (PKC) (40–42). PKCα and PKCδ are abundant in keratinocytes and have been implicated as regulators of differentiation (43). Previous studies suggest that calcium regulates involucrin gene expression at the mRNA and protein levels (44, 45), and PKCδ was recently reported to enhance the calcium-dependent activation of involucrin promoter activity and endogenous gene expression (46). It is not known whether DDV10 gene expression is regulated by calcium, and there are no data concerning the biological role of DDV10 in epithelial differentiation. DDV10 is a calcium-dependent, carbohydrate-binding protein; hence, it could play an important role in epithelial differentiation via cell-cell interactions and/or signal transduction. This is the first evidence for the existence of an estrogen-regulated C-type lectin in vaginal epithelial cells, and we hypothesize that DDV10 may play an important role in regulating the estrogen-induced differentiation of vaginal epithelium in cooperation with calcium. Therefore, future studies aimed at delineating the biological function of DDV10 and its regulation by E2 will improve our understanding of how E2 regulates epithelial differentiation.

Acknowledgments

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Estrogen Receptors (α and β) and 17β-Hydroxysteroid Dehydrogenase Type 1 and 2 in Thyroid Disorders: Possible *In Situ* Estrogen Synthesis and Actions

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Both epidemiological and experimental findings suggest the possible roles of sex steroids in the pathogenesis and/or development of various human thyroid disorders. In this study, we evaluated the expression of estrogen receptors (ER) α and β in normal thyroid glands ($N = 25$; female: $n = 13$, male: $n = 10$, unknown: $n = 2$) ranging in age from fetus to adult. Furthermore, using immunohistochemistry, we investigated the expression of ERα and β in 206 cases of thyroid disorders, including 24 adenomatous goiters, 23 follicular adenomas, and 159 thyroid carcinomas. In addition, we also studied the mRNA expression of ERα and β and 17β-hydroxysteroid dehydrogenase Type 1 and 2, enzymes involved in the interconversion between estrone and estradiol, using reverse transcription polymerase chain reaction (RT-PCR), in 48 of these 206 cases (10 adenomatous goiters, 10 follicular adenomas, and 28 papillary thyroid carcinomas) in which fresh frozen tissues were available for examination to further elucidate the possible involvement of intracrine estrogen metabolism and/or actions in thyroid disorders. ERα labeling index, or percentage of cells immunopositive for ERα, was significantly higher in adenomatous goiter (14.2 ± 6.4), follicular adenoma (13.4 ± 5.1), and thyroid carcinoma (16.4 ± 2.1) than in normal thyroid gland (0; $P < .05$). Few follicular cells were positive for ERα in normal thyroid glands. In papillary carcinoma, ERα labeling index was significantly higher in premenopausal women (28.1 ± 4.5) than in postmenopausal women (14.2 ± 2.9) and in men of various ages (7.6 ± 2.7; $P < .05$). In other histological types of thyroid carcinoma, no significant correlations were detected. ERβ immunoreactivity was detected in both follicular and C-cells of normal thyroid glands, including those in developing fetal thyroid glands. In addition, ERβ immunoreactivity was detected in the nuclei of various thyroid lesions. But no significant correlations were detected between ERβ labeling index and clinicopathological findings including age, menopausal status, gender, and/or histological type of thyroid lesions. 17β-hydroxysteroid dehydrogenase Type 1 expression was detected in 31/48 (64.0%) of the cases examined, whereas Type 2 was detected only in 3/46 (6.3%) of all the cases examined. These results demonstrated that estrogens may influence the development, physiology, and pathology of human thyroid glands, and these effects, especially through ERα, may become more pronounced in neoplasms, particularly in papillary carcinoma arising in premenopausal women.

KEY WORDS: Estrogen receptors, Human thyroid lesions, 17β-hydroxysteroid dehydrogenase, Immunohistochemistry, Labeling index, Reverse transcription polymerase chain reaction.

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Biological effects of estrogens are generally mediated through an initial interaction with the estrogen receptor (ER), a member of the superfamily of nuclear receptors. Identification of ER is an initial step in understanding the estrogenic effects on various

tumors. ER has been identified, for instance, in a wide range of human neoplasms, including carcinoma arising in colon (1), lung (2), pancreas (3), and other organs classically considered not to be targets for estrogens. However, their biological significance in these tumors has not been fully elucidated. Thyroid tumors are well known to occur with approximately three times more frequency in women than in men (4). Given the higher incidence and relatively better prognosis of thyroid carcinomas diagnosed in women (5), especially in premenopausal women, it is reasonable to speculate that these thyroid lesions may be influenced by sex hormones, especially estrogens, in their pathogenesis and/or development.

In addition to the classical ERα isoform, a second isoform of ER or ERβ, has been recently identified in humans (6). ERβ has been demonstrated to be widely distributed in various human tissues (7, 8), including many organs of the human fetus (9, 10) in contrast to ERα. Several investigators have demonstrated the presence of ERα in thyroid tissues from human thyroid tumors and normal thyroid glands (11–13), but not that of ERβ. In addition, Valle *et al.* (11) reported an overexpression of aromatase, the enzyme involved in conversion of androgens to estrogens, in several human thyroid lesions. However, results of these studies on ERα in normal and pathologic human thyroids have been inconsistent and could not necessarily account for the marked prevalence of thyroid lesions among female subjects. 17β-Hydroxysteroid dehydrogenase catalyzes the reversible interconversion between estrone and estradiol. However, 17β reduction and oxidation of estrogens is catalyzed by different 17β-hydroxysteroid dehydrogenase isozymes. 17β-hydroxysteroid dehydrogenase Type 1 mainly catalyzes the conversion of inactive estrogen, estrone, to the biologically active estrogen, estradiol (14–16), whereas 17β-hydroxysteroid dehydrogenase Type 2 predominantly catalyzes the conversion of estradiol to estrone (17). 17β-hydroxysteroid dehydrogenase Type 1 and 2 both are known to regulate the *in situ* levels of estradiol, and subsequently to modulate estrogenic actions in estrogen target tissues. Examination of 17β-hydroxysteroid dehydrogenase Type 1 and 2 in human thyroid lesions, in addition to aromatase and ER isoforms, therefore, become very important in obtaining a better understanding of the local or intracrine regulation of estrogenic actions in human thyroid lesions. Therefore, in this study, we first examined the expression of ERα and β in normal thyroid glands obtained from fetal, pediatric, and adult patients. We then studied the expression of ERα and β, and 17β-hydroxysteroid dehydrogenase Types 1 and 2 in adenomatous goiters ($n = 24$), follicular adenomas ($n = 23$), and thyroid carcinomas ($n = 159$), using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) to elucidate the possible roles of estrogens in the development, physiology, and pathology of the human thyroid gland.

MATERIALS AND METHODS

Tissue Preparation

Human fetal tissues (10–21 weeks gestational age) were obtained after elective termination in normal pregnant women at Tohoku University Hospital and Nagaike Maternal Clinic (Sendai, Japan). Informed consent was obtained from these pregnant women before elective termination. The ages of the fetuses were estimated by the last menstrual date, body weight, and/or crown-rump length. Human fetal tissues of 24, 27, 37, and 39 weeks of age were obtained at the time of autopsy at Tohoku University Hospital. The ethics committee of Tohoku University School of Medicine approved this research protocol. The time elapsed from demise to the removal of the tissues ranged from 30 minutes to 1 hour in the case of elective termination, and from 2 to 6 hours in autopsy cases. After careful evaluation, these specimens did not appear to have any significant histopathological abnormalities. The specimens for immunohistochemistry were fixed in 10% neutral formalin for 18 hours at 4° C and then embedded in paraffin.

Patients and Tissues

Paraffin-embedded blocks from 206 patients who underwent thyroidectomy from 1990 to 2000 at Tohoku University Hospital, Sendai City Hospital, and Itou Hospital (Tokyo, Japan) were used for immunohistochemical studies. The mean age of the patients was 52.8 ± 17.5 years old (range, 12–86 y). Twenty-four cases of adenomatous goiter (18 females, 6 males; 53.5 ± 1.0 y), 23 cases of follicular adenoma (17 females, 6 males; 48.1 ± 14.9 y), and 159 cases of carcinoma (116 females, 43 males; 53.8 ± 17.5 y) were retrieved from the surgical pathology files at all three hospitals mentioned above. Carcinoma cases that we examined included 100 papillary (79 females, 21 males; 53.5 ± 17.6 y), 14 follicular (8 females, 6 males; 47.6 ± 17.5 y), 25 anaplastic (15 females, 10 males; 65.2 ± 9.1 y), and 20 medullary (14 females, 6 males; 45.7 ± 18.6 y) carcinomas (Table 1). All patients examined in this study received neither irradiation nor chemotherapy before surgery. The World Health Organization classification of thyroid lesions and the general rules for the description of thyroid cancer in Japan (18) were used as the basis for histological classification of thyroid tumors investigated in this study.

TABLE 1. Histological Diagnosis of Paraffin-Embedded Thyroid Tissues

	Women		Men	Total
	Premenopausal	Postmenopausal		
Normal	3	3	5	11
Adenomatous goiter	7	11	6	24
Follicular adenoma	10	7	6	23
Carcinoma	47	69	43	159
Papillary	35	44	21	100
Follicular	5	3	6	14
Anaplastic	0	15	10	25
Medullary	7	7	6	20
Total	67	91	59	217

Fresh thyroid tissues were available for total RNA extraction and RT-PCR examination in 48 patients with thyroid disease from 1997 to 2000 at the three institutions above. Thyroid tissues were immediately frozen in liquid nitrogen and stored at −80° C until use for the RT-PCR study. The mean age of the patients was 53.3 ± 15.5 years (range, 20–86 y). These 48 cases included 28 papillary carcinomas (21 females, 7 males; 55.8 ± 16.2 y), 10 follicular adenomas (8 females, 2 males; 47.2 ± 14.6 y), and 10 adenomatous goiters (9 females, 1 males; 52.7 ± 13.9 y). All of these cases were histologically confirmed.

Antibodies

The polyclonal antibody for ERβ was raised in rabbit against synthesized peptides of the C-terminal region of ERβ (CSPAEDSKSKEGSQN-PQSQ). This antibody was purified on affinity columns bound with the synthetic peptide. The characterization of this antibody was confirmed by Western blotting (19), and use of the ERβ antibody for immunohistochemistry has been previously reported. The monoclonal antibody for ERα (ER1D5) was purchased from Immunotech (Marseille, France).

Immunohistochemistry

In this study, immunohistochemical analyses were performed using the streptavidin-biotin amplification method and a Histofine Kit (Nichirei, Tokyo, Japan) for ERα, and Envision+ (DAKO, Carpinteria, CA) for ERβ. For antigen retrieval, slides were deparaffinized and heated in an autoclave at 120° C for 5 minutes in a citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for the immunostaining of ERα and ERβ. The dilutions of primary antibodies used in this study were as follows: ERα 1/100, and ERβ 1/100. The antigen–antibody complex was visualized with 3.3-diaminobenzidine (3,3'-diaminobenzidine) solution M 3,3'-diaminobenzidine, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2, and counterstained with hematoxylin. Breast cancer was used as a positive control for ERα, and normal breast was also used as a positive control for ERβ. Normal rabbit or mouse IgG was used instead of the primary antibodies as a negative control. No specific immunoreactivity was detected in these sections.

RT-PCR Analysis

Total RNA was extracted by homogenizing tissue specimens in guanidium thiocyanate, followed by ultracentrifugation in cesium chloride. Total RNA was spectrophotometrically quantified at 260 nm. An RT-PCR kit (SUPERSCRIPT Preamplification system, Gibco-BRL, Grand Island, NY) was employed in the synthesis and amplification of cDNA. cDNAs were synthesized from 5 μg of total RNA using random hexamer primer. Reverse transcription was carried out for 60 minutes at 42° C with SUPERSCRIPT II reverse transcriptase. After an initial 1-minute denaturation step at 95° C, 40 cycles of PCR were carried out on a Light Cycler PCR machine (Roche Diagnostics GmbH, Mannheim, Germany) under the following condition: 0 seconds of denaturation at 95° C, 15 seconds of annealing at 62° C for ERα or at 60° C for ERβ and GAPDH, followed by a 15-second extension at 72° C. The primer sequences used in this study are listed in Table 2 (21–24). After PCR, the products were resolved on a 2% agarose ethidium bromide gel, and images were captured with Polaroid film under ultraviolet transillumination. In initial experiments, amplified PCR products were purified and subjected to direct sequencing (ABI PRISM Biosystems, Foster City, CA; and ABI Prism 310 Genetic Analyzer) to verify amplification of the correct sequences. As a positive control, T-47D breast cancer cells were used for ERα and ERβ; frozen placental tissue was used as a positive control for 17β-HSD Type 1 and 2. In the present study, we tested for the presence of exogenous and/or non-gene-specific contaminant DNA by synthesizing cDNA in the absence of reverse transcriptase and also by performing a standard PCR without cDNA substrate. We observed no amplification of gene-specific products under either of these conditions.

Scoring of Immunoreactivity

Scoring of ERα and ERβ in tumor cells was performed on high-power field (400×) using a standard light microscope. In each case, the fields for examination were simultaneously determined by two of the authors (WK and TS) using a double-headed light microscope. More than 1000 tumor cells were counted independently by the two authors above. Nuclear immunoreactivity was considered positive regardless of immunointensity. The

TABLE 2. Primer Sequences Used in RT-PCR Analysis

cDNA	Sequence	Position in cDNA	Size (bp)	Reference Number
ERα	Forward 5'AAGAGCTGCCAGGCCTGCC	702–720	167	21
	Reverse 5'TTGGCAGCTCTCATGTCTCC	850–869		
ERβ	Forward 5' GCATGGAACATCTGCTCAAC	1513–1532	229	22
	Reverse 5' ACGCTTCAGCTTGTGACCTC	1721–1740		
17β-HSD type 1	Forward 5'CTCTGGGCTGCCCCAACAC	598–615	352	23
	Reverse 5'GGACGTGCTGGTGTGTAAC	264–282		
17β-HSD type 2	Forward 5'CTTTGTGACCTCCACAGTTC	667–686	418	23
	Reverse 5'GGTGTCATGCTTCCTCATGT	269–288		
GAPDH	Forward 5'TGAACGGGAAGCTCACTGG	731–750	307	24
	Reverse 5' TCCACCACCCTGTTGCTGTA	1018–1038		

percentage of positive immunoreactivity, that is, labeling index, was determined. When interobserver differences were >5%, the cases were simultaneously re-evaluated using a double-headed light microscope. When interobserver differences were <5%, the mean values of these two observed data were determined as the labeling index for that case. Interobserver differences in our study were <1%.

Statistical Analysis

The gender and menopausal status of the patients in this study were tentatively classified into three groups (premenopausal women, postmenopausal women, and men of various ages). Values for each category and the labeling index for ERα and ERβ are presented as mean ± standard error of means (SEM). Associations between the labeling index for ERα and ERβ and each histological category were evaluated using an ANOVA test. A *P*-value of <.05 was considered significant.

RESULTS

Immunohistochemistry

Results are summarized in Tables 3 and 4.

ERα

ERα immunoreactivity was detected in the nuclei of invasive ductal carcinoma employed as positive control of immunostain. Very few follicular cells were positive for ERα in the normal thyroid gland of both women and men. ERα immunoreactivity was not detected in the nuclei of follicular cells in developing fetal thyroid glands. However, ERα immunoreactivity was detected in the nuclei of parenchymal cells in thyroid disorders (Fig. 1, A–D). The numbers of ERα-positive cases were as follows: 9/24 (37.5%: 6/18 [33.3%] females, 3/6 [50%] males) in adenomatous goiters, 6/23 (26.1%: 4/17 [23.5%] females, 2/6 [33.3%] males) in follicular adenomas, and 74/159 (46.5%: 58/116 [50.0%] females, 16/43 [37.2%] males) in carcinomas. ERα labeling index or the percentage of ERα-positive cells was as follows: 14.2 ± 6.4 in adenomatous goiter, 13.4 ± 5.1 in follicular adenoma, 17.8 ± 2.7 in papillary carcinoma, 13.3 ± 6.3 in follicular carcinoma, 18.8 ± 5.7 in anaplastic carcinoma, and 5.5 ± 3.8 in medullary carcinoma.

Results of the correlation between ERα labeling index and clinicopathological parameters are summarized in Table 3. ERα labeling index was significantly higher in adenomatous goiter (14.2 ± 6.4), follicular adenoma (13.4 ± 5.1), and thyroid carcinoma (16.4 ± 2.1%) than in normal thyroid gland (0; $P < .05$). In papillary carcinoma, ERα labeling index was significantly higher in premenopausal women (28.1 ± 4.5) than in postmenopausal women (14.2 ± 2.9) and men of various ages (7.6 ± 2.7; $P < .05$). With respect to anaplastic carcinoma, ERα labeling index tends to be higher in men of various ages (34.7 ± 10.1) than in postmenopausal women (8.2 ± 5.4), but the differences did not reach statistical significance. No significant correlations were detected in other histological types. Clinical

TABLE 3. Mean ± SEM of Labeling Indexes of ERα Immunoreactivity

	Women		Men	Total
	Premenopausal	Postmenopausal		
Normal	0	0	0	0
Adenomatous goiter	8.2 ± 6.9	10.5 ± 6.3	23.2 ± 13.0	14.2 ± 6.4
Follicular adenoma	6.8 ± 4.8	23.5 ± 4.1	11.8 ± 10.8	13.4 ± 5.1
Carcinoma				
Papillary	28.1 ± 4.5	14.2 ± 2.9	7.6 ± 2.7	17.8 ± 2.7
Follicular	20.3 ± 10.5	14.3 ± 14.3	21.8 ± 10.4	13.3 ± 6.3
Anaplastic	—	8.2 ± 5.4	34.7 ± 10.1	18.8 ± 5.7
Medullary	6.3 ± 5.5	10.2 ± 9.4	0	5.5 ± 3.8

TABLE 4. Mean ± SEM of LIs of ERβ Immunoreactivity

	Women		Men	Total
	Premenopausal	Postmenopausal		
Normal	12.5 ± 2.6	13.0 ± 3.9	21.6 ± 3.0	22.4 ± 2.5
Adenomatous goiter	17.2 ± 14.5	12.2 ± 7.4	16.2 ± 9.2	15.2 ± 7.3
Follicular adenoma	29.0 ± 5.2	23.5 ± 10.8	34.0 ± 19.0	29.2 ± 8.2
Carcinoma				
Papillary	15.4 ± 4.5	17.2 ± 3.2	19.6 ± 6.2	17.0 ± 2.9
Follicular	12.5 ± 11.2	23.3 ± 13.6	13.6 ± 6.2	15.5 ± 5.3
Anaplastic	—	38.4 ± 7.1	42.0 ± 9.5	39.9 ± 5.6
Medullary	7.2 ± 2.7	40.5 ± 16.2	24.3 ± 13.7	24.0 ± 7.9

follow-up data of these patients with thyroid carcinoma also were not available for present study.

ERβ

ERβ nuclear immunoreactivity was detected widely in both follicular or parenchymal and C-cells of normal thyroid glands, including those in the developing fetal thyroid glands (Fig. 1, E–F). In addition, ERβ immunoreactivity was detected in the nuclei of various thyroid lesions. The number of ERβ-positive cases or percentages of ERβ-positive cases were as follows: 9/24 (37.5%: 6/18 [33.3%] females, 3/6 [50%] males) in adenomatous goiters, 6/23 (26.1%: 4/17 [23.5%] females, 2/6 [33.3%] males) in follicular adenomas and 74/159 (46.5%: 70/116 [60.3%] females, 25/43 [58.1%] males) in carcinomas. ERβ labeling index was as follows: 17.7 ± 4.6 in normal thyroid gland, 15.2 ± 7.3 in adenomatous goiter, 29.2 ± 8.2 in follicular adenoma, 17.0 ± 2.9 in papillary carcinoma, 15.5 ± 5.3 in follicular carcinoma, 39.9 ± 5.6 in anaplastic carcinoma, and 24.0 ± 7.9 in medullary carcinoma.

Results of the correlation between ERβ labeling index and clinicopathological parameters of patients were summarized in Table 4. ERβ labeling index tended to be higher in anaplastic carcinoma (39.9 ± 5.6) than in other histological types, but the differences did not reach statistical significance. There were no significant correlations between ERβ labeling index and patient age, menopausal status, gender, benign and malignant tumors, histological type and follow-up data (data not shown).

RT-PCR

Messenger RNA (mRNA) expression for ERα, ERβ, and 17β-hydroxysteroid dehydrogenase Type 1 and 2 was detected as a signal gene-specific band (168 bp for ERα, 228 bp for ERβ, 352 bp for 17β-hydroxysteroid dehydrogenase Type 1, and 418 bp for 17β-hydroxysteroid dehydrogenase Type 2; Fig. 2) in 48/48 (100%), 48/48 (100%), 31/48 (64.6%), and 3/48 (6.3%) specimens examined, respectively. Results of RT-PCR analysis were consistent with those of immunohistochemistry in each case.

No significant correlations were detected between 17β-hydroxysteroid dehydrogenase Type 1 and the labeling index for ERα and β, or between 17β-hydroxysteroid dehydrogenase Type 2 and the labeling index for ERα and β.

DISCUSSION

In our present study, ERα immunoreactivity was not detected in the normal thyroid gland, but ERβ immunoreactivity was detected in the nuclei of follicular epithelial cells and C-cells throughout the development of the normal thyroid gland. The presence of ERβ previously has been demonstrated in the normal rat thyroid gland by immunohistochemistry (24). In addition, ERβ has very recently been demonstrated in adult normal human thyroid gland by immunohistochemistry (8). Results from our present study are also consistent with those from previous studies. In our study, ERβ immunoreactivity was detected throughout the development of the human thyroid gland from 11 gestational weeks. In normal pregnancy, large quantities of estrogens are produced by placental syncytiotrophoblasts, which subsequently move into both the fetal and maternal circulation. The effects of estrogens on thyroid development have not been well studied, but results of our present study suggest that estrogens may also be involved in the development of fetal thyroid glands. These effects are most likely to be predominantly mediated via ERβ.

ERα immunoreactivity was detected in the nuclei of thyroid parenchymal cells of various thyroid lesions. The presence of ERα has previously been examined in human thyroid neoplasms by a number of investigators (11–13, 25–27). However, results from these studies were inconsistent, possibly because of the different methods employed. In our present study, we employed immunohistochemistry in combination with labeling index, one of the most reliable methods for detecting ERα in clinical specimens, and also RT-PCR. ERα labeling index was significantly higher in adenomatous goiters, follicular adenomas and thyroid carcinomas than in normal thyroid glands. In the papillary carcinoma,

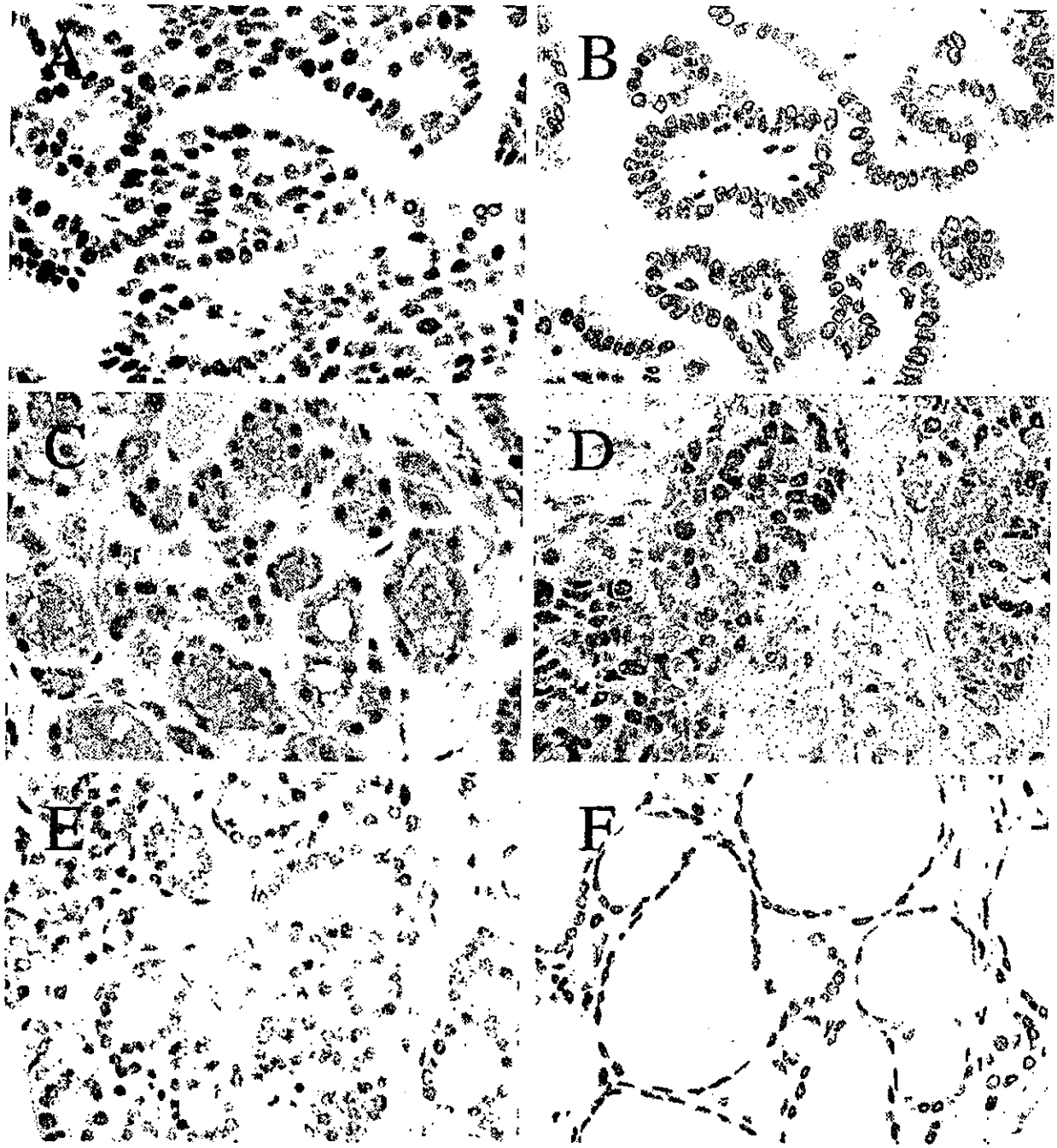

FIGURE 1. Immunohistochemistry for ERα in (A, B) papillary carcinoma, (C) adenomatous goiter obtained from premenopausal women, respectively, and (D) anaplastic carcinoma obtained from postmenopausal women. Immunohistochemistry for ERβ in (E) the fetal thyroid gland (14 gestational weeks) and (F) in the adult thyroid gland.

ERα labeling index was significantly higher in postpuberty/premenopausal women than in postmenopausal women and men of various ages. A statistically significant association between ERα and the age of the patients was not detected in other types of thyroid lesions. Estradiol has been demonstrated to stimulate cell proliferation of FRTL-5 rat cells, a well-established cell line derived from thyroid papillary carcinoma (28), and the human thyroid papillary carcinoma cell line, HTC-TSHr (29). These results all suggest that higher estrogen concentrations in the serum may be responsible for the relatively high incidence of thyroid papillary carcinoma in premenopausal women. In addition, a higher level of ERα labeling index may also be involved in the activation of

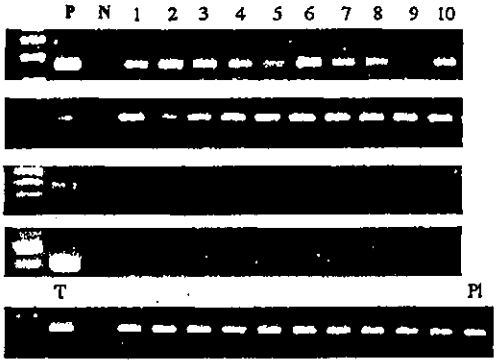

FIGURE 2. RT-PCR analysis for ERα, ERβ, and 17β-HSD Type 1 and Type 2 in human thyroid tumors. mRNA expression for ERα, ERβ, 17β-HSD Type 1, Type 2, and GAPDH were detected as a single gene-specific band (168 bp for ERα, 228 bp for ERβ, 352 bp for 17β-HSD Type 1, 418 bp for 17β-HSD Type 2 and 307 bp for GAPDH). P, Positive controls (T-47D for ERα and ERβ, placenta for 17β-HSD Type 1 and Type 2); T, T-47D; Pl, placenta; N, negative control (no cDNA substrate). All thyroid lesion specimens were obtained from (pre-) or (post-) menopausal women with the exception of 6 and 10, which were obtained from male patients. 1–2, adenomatous goiter (post-); 3–4, papillary carcinoma (pre-); 5, adenomatous goiter (pre-); 6, papillary carcinoma; 7, follicular adenoma (pre-); 8, adenomatous goiter (pre-); 9, papillary carcinoma (post-); 10, papillary carcinoma.

tumorigenesis of papillary carcinoma in premenopausal women, but it awaits further investigations for clarification.

The status of ERβ has not been well studied in human thyroid disorders. In our present study, ERβ nuclear immunoreactivity was detected relatively widely in thyroid neoplastic parenchymal cells. There were, however, no significant correlations between ERβ immunoreactivity and any clinicopathological parameters examined, including patient age, menopausal status, gender, histological type, and fetal developmental stage. In addition, ERβ is widely distributed in follicular cells and C-cells of the normal thyroid gland throughout development. These results above suggest that estrogenic effects via ERβ play important roles in the maintenance of homeostasis of thyroid follicles, and possibly of thyroid hormone biosynthesis.

P450 aromatase converts androstenedione to estrone and testosterone to estradiol. Recent studies demonstrated that bioactive estrogen, estradiol, is produced locally in breast carcinoma (30). Estrogen also has been reported to be produced locally in human thyroid tumors (11). In addition, aromatase immunoreactivity has been demonstrated to be expressed in the cytoplasm of follicular epithelial cells (11). In our present study, the expression of 17β-hydroxysteroid dehydrogenase Types 1 and 2 was demonstrated by RT-PCR. Especially, 17β-hydroxysteroid dehydrogenase Type 1 was detected in 31/48 (64.0%) of the cases examined. Expression of ER, aromatase, and 17β-hydroxysteroid dehydrogenase Type 1 has been demonstrated in human thyroid lesions as in human breast carcinoma (30). This mechanism can effectively produce estrogens *in situ* from circulating androgens and exert their effects locally. Further investigations are required to fully elucidate the roles and mechanisms of estrogenic actions with respect to pathogenesis and/or development of human thyroid disorder.

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Aggressive Behavior and Serum Testosterone Concentration during the Maturation Process of Male Mice: The Effects of Fetal Exposure to Bisphenol A

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The relationship between exposure to endocrine-disrupting chemicals (EDs) and risk to reproductive organs is well documented, but the influence of EDs on behavioral development has not been studied. In this study we evaluated the effect of fetal exposure to bisphenol A, which mimics estrogenic activity, on aggressive behavior and hormonal change in male mice. On gestation days 11–17, female mice were fed bisphenol A at 2 ng/g or 20 ng/g of body weight (environmentally relevant concentration). Aggression rating and blood sampling of the offspring were done at 8, 12, and 16 weeks of age. Aggression scores increased significantly ($p < 0.01$) at 8 weeks of age in male mice exposed to bisphenol A at both the 2 ng/g and 20 ng/g concentrations compared with a control group, but no difference was found after 12 weeks. Relative testis weight (per gram of body weight) was significantly lower at 8 and 12 weeks in mice treated with 2 ng/g than in controls ($p < 0.05$) and was significantly lower at 12 weeks in mice treated with 20 ng/g than in controls ($p < 0.01$). The serum testosterone concentration in treated mice was not significantly different from that in controls. These results demonstrate that bisphenol A temporarily activated aggressive behavior in mice at 8 weeks of age and that low doses of bisphenol A interfered with the normal development of reproductive organs. The mechanism activating this aggressive behavior was not elevated testosterone concentration. *Key words:* aggression, behavior, bisphenol A, mice, sexually mature, testosterone. *Environ Health Perspect* 111:175–178 (2003). [Online 1 October 2002]
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Alterations in the normal pattern of reproductive development seen in some populations of wildlife have been linked to exposure to endocrine-disrupting chemicals (EDs), which have been shown to mimic sex steroid hormones (*1*). Sex steroids exert potent influences on the nervous system during the critical development periods of the neuroendocrine system and behavior (*2,3*).

Palanza et al. (*4*) reported that prenatal exposure to the estrogenic chemical diethylstilbestrol (DES) increased the frequency of both males and females that responded aggressively to a same-sex conspecific. In addition, in other studies aggressive behavior was reduced and male-typical offensive attacks were rarely displayed by transgenic estrogen-receptor–deficient mice (*5,6*). However, few studies have focused on the influence of EDs on behavior (*4,7,8*). The relationship between aggressive behavior during the maturation process and influence of bisphenol A, which is one of most common EDs, has never been examined. Bisphenol A is a monomer used in the manufacture of the resin used to line food and drink cans and from which polycarbonate plastic is made (*9,10*). Bisphenol A is also a component of the plastic used in dental fillings (*9,10*).

In social species such as mice, expression of aggression is important for determining the reproductive success and regulation of the density of animals according to ecologic conditions (*11*). The purpose of this study was to investigate the influence of bisphenol A on aggression during the maturation process in male mice.

Materials and Methods

Animals. Pregnant CD-1 mice were purchased from KBT Oriental (Saga, Japan). Pregnant mothers were separated into individual, standard polypropylene mouse cages, where they remained undisturbed throughout pregnancy. The mice were maintained under standard, controlled conditions (temperature, 21 ± 1°C; relative humidity, 60%; lights on from 800 to 2000 hr) and had free access to food and water. This experiment was approved by the Committee of Ethics in Animal Experiments of the Faculty of Medicine, Kyushu University, Fukuoka, Japan, and the law (No. 105) and notification (No. 6) of the Government of Japan.

Treatment of pregnant females with bisphenol A. The treatment protocol of this study is shown in Figure 1. We followed the protocol previously reported by vom Saal et al. (*12*). Briefly, bisphenol A dissolved in corn oil (catalog no. 25606-65, Nacalai Tesque, Kyoto, Japan) was fed to pregnant female mice (n = 30) once per day (at 1000 hr) on gestation days 11–17. Pregnant mothers were separated into individual cages on day 7 of pregnancy. The pregnant mothers were randomly selected for treatment. The dose for the two treatment groups and the controls throughout the 7 days of treatment was 2 ng/g body weight (n = 7), 20 ng/g body weight (ppb) (n = 7), and 0 ng/g (n = 9) body weight, respectively, all within the range of current human exposure (*12*). The body weight of each mouse was measured three times a week for determination of treatment dose. A micropipette enabled delivery of an accurate volume of corn oil into the mouth.

The date of birth was designated day 0. Litters were housed with their mothers in plastic cages. Pups were weaned on day 21. Each litter had four to seven male pups. Randomly selected males from the same litter were housed in groups of four or five. The mice were maintained under standard, controlled conditions described above.

Study design. Clean plastic containers (17 × 28 × 13 cm) were used for aggression tests (*13*). We randomly selected 15 male mice from the nine cages of control mice to serve as opponents. These opponents were housed in three cages (five mice each). Each opponent was used for testing only once daily. The male mouse to be tested was placed alone in a neutral container for 5 min. The opponent mouse, age matched with the resident test animal, was introduced into the cage. The behavior of the resident mouse was recorded over a 7-min encounter with the opponent. This assessment included the cumulative time that the tested mouse had contact with the opponent after approaching of its own accord. This measurement included body contact by sniffing or attacking. Sniffing included exploration of the head, flanks, and anogenital region. Cumulative times were recorded by staff members trained in this method and who were not familiar with the treatment histories of the subject mice. Frequent vigorous sniffing and attacking indicated a high degree of aggression according to the report of Lagerspetz and Hautojarvi (*13*).

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The aggression test was performed when the mice were 8, 12, and 16 weeks of age. Mice not sacrificed until 16 weeks were tested a total of three times. Testing was done under artificial white light between 1400 and 1800 hr. The mice were maintained under standard, controlled conditions described above.

Blood collection, hormone assay, and testis weight. Randomly selected mice, 8–14 mice in each group, were sacrificed 1 week after aggression testing (ages 9, 13, and 17 weeks), and trunk blood was collected for the measurement of testosterone. Testosterone was assayed in each mouse serum sample using single commercial radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA, USA). Body and testis weight were recorded at the time of sacrifice.

Statistics. Data are shown as mean ± SE. Treatment comparisons were performed by analysis of variance (StatView, version 5; SAS Institute, Cary, NC, USA). We calculated the correlation between contact time and testosterone concentration using the Spearman rank correlation test (StatView). A p-value of < 0.05 was chosen as the level of significance.

Results

Chronologic change of relative testis weight. We found no significant differences in body weight among the groups during the experiment (data not shown). Figure 2 shows the change in testis weight per body weight of male control mice and male offspring of pregnant females fed bisphenol A at 2 or 20 ng/g body weight. The relative testis weight of the 2-ng/g bisphenol A group remained low throughout the test. Relative testis weight was significantly lower in the 2-ng/g bisphenol A group than in the controls at 8 and 12 weeks (6.2 ± 0.2 vs. 6.9 ± 0.3, 6.2 ± 0.2 vs. 7.6 ± 0.3; $p = 0.047$, $p < 0.0015$, respectively). Relative testis weight was significantly lower in the 20-ng/g bisphenol A group than in the controls at 12 weeks (6.8 ± 0 vs. 7.6 ± 0.8; $p = 0.04$).

Chronologic change of aggressive behavior. Figure 3 shows differences in contact time between the male control mice and the 2-ng/g bisphenol A and 20-ng/g bisphenol A male offspring. Contact time in the control mice gradually increased from weeks 8 to 12. In contrast, the treated mice had high aggression scores at 8 weeks of age, after which aggression diminished.

Aggression scores (contact time) were significantly increased at 8 weeks of age in the 2-ng/g bisphenol A (43.7 ± 4.0 sec; $p = 0.003$) and 20-ng/g bisphenol A (48.0 ± 6.5 sec; $p = 0.0018$) groups compared with the control group (19.5 ± 3.3; $p = 0.003$), but no difference was found among these groups at 12 and 16 weeks. In this experiment, no mice showed any indication of attacking behavior.

Chronologic change of serum testosterone concentration. Figure 4 shows the change of serum testosterone concentration in male control mice and the 2-ng/g bisphenol A and 20-ng/g bisphenol A groups. The serum testosterone concentration was lower at 12 weeks of age in both the 2-ng/g bisphenol A and 20-ng/g bisphenol A groups than in the controls, but the differences were not significant.

Figure 5 shows the correlation between contact time and testosterone concentration at 8 weeks of age.

We found no significant correlation between contact time and testosterone concentration at 8 weeks of age in either of the treatment groups (2-ng/g bisphenol A and 20-ng/g bisphenol A) or the control group.

Discussion

This study shows three important features of prenatal exposure to bisphenol A in mice: *a*) behavioral change is caused by this estrogen-mimicking substance; *b*) treatment with bisphenol A at an early stage of life significantly decreased relative testis weight; and *c*) a low dose of bisphenol A apparently had a larger effect on relative testis weight than did the much higher dose of 20 ng/g.

Behavioral change. Behavioral change is caused by this estrogen-mimicking substance. Male mice exposed to bisphenol A during fetal development, a period of differentiation of organs and testosterone secretion, showed a temporary, high aggression score at 8 weeks of age, the approximate age when mice usually reach sexual maturity. A possible weakness of this study is that we did not test younger, more sexually immature mice for comparison.

It is well documented that one of the most important factors in the induction of male patterning in the central nervous system (CNS) is estrogen converted from testosterone originating in the testis during the critical period of sexual differentiation (*3,14*). In females, endogenous estrogen from the ovaries cannot move to the brain because of the action of estrogen-binding α-fetoproteins (*3,15*). Thus, estrogen plays an important role in CNS sexual differentiation in males. The critical period of CNS sexual differentiation in mice has been reported to be postnatal (*3,15*). It is not clear whether bisphenol A, which has an exogenous estrogen-mimicking activity, is affected by α-fetoproteins. As the experimental results presented here show, bisphenol A might affect the development of male patterning in the CNS and aggressive behavior during the growth process. Furthermore, it is well documented that aggressive and sexual behaviors in males can be affected by testosterone, which acts not only through androgen receptors but also through estrogen receptors (ERs)

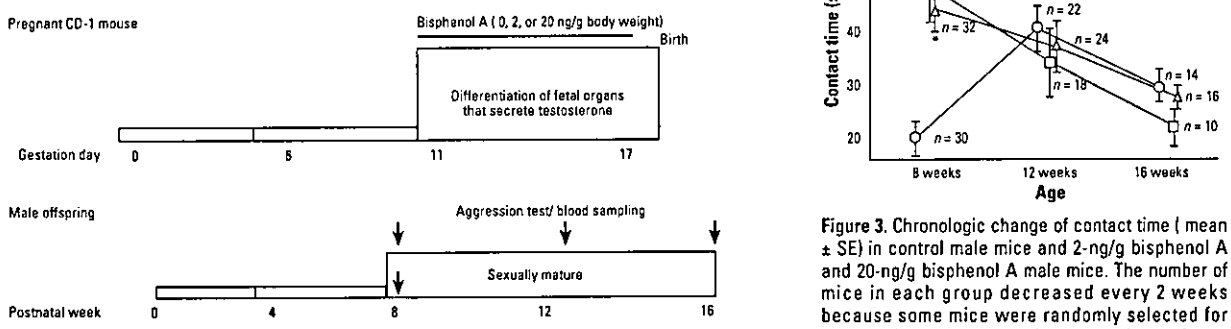

Figure 1. Experimental design indicating the period of bisphenol A treatment (2 or 20 ng/g body weight) of pregnant mice (black bar) and aggression testing (arrows) of male offspring prenatally exposed to bisphenol A.

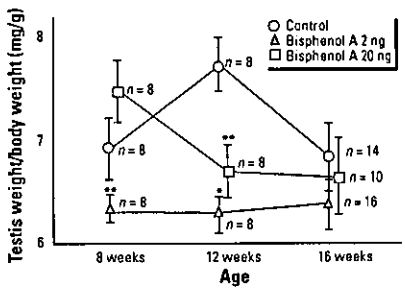

Figure 2. Chronologic change of relative testis weight per body weight (mean ± SE) in control male mice and 2-ng/g bisphenol A and 20-ng/g bisphenol A male mice.
*$p < 0.01$ compared to control. **$p < 0.05$ compared to control.

Figure 3. Chronologic change of contact time (mean ± SE) in control male mice and 2-ng/g bisphenol A and 20-ng/g bisphenol A male mice. The number of mice in each group decreased every 2 weeks because some mice were randomly selected for sacrifice from each group after aggression testing.
*$p < 0.01$ compared to control.

(*5,6,16*). Lack of ER-α has been shown to suppress male-typical offensive attacks (*5,6*). However, we were unable to establish any relationship between temporary aggression in young male mice and serum testosterone concentration at 8 weeks of age. Because the surge of gonadal hormones occurs from approximately 8 weeks, along with the process of sexual maturation, we speculate that sensitivity to gonadal hormones might have been changed in our treated mice and thus caused marked changes in aggressive behavior. The first test was done at 8 weeks of age in our study, but a future study of 4–6-week-old mice will be necessary to clarify in this point.

Of question is how to explain temporary aggression in male mice. The results of this study are insufficient to explain this problem. Gorski and colleagues (*3,17*) reported that the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in the rat hypothalamus is several times larger in males than in females. They advanced the theory that gonadal steroids can stimulate SDN-POA development directly in female rats and that regulation of apoptosis by testosterone is one mechanism involved in the sexual differentiation of the SDN-POA (*18,19*). The histologic data of the present study will be addressed in future analyses. Other factors that were not considered, such as gonadal hormone receptors and the postreceptor area, may have been involved. As mentioned in the "Materials and Methods," the doses of bisphenol A used were environmentally relevant. For example, during the first hour after application of dental sealant, Olea et al. (*9*) detected in saliva 931 μg of bisphenol A, the monomer used to make the plastic polymer. For a person weighing 70 kg, this represents 13.3 (13.3 ppb), and for an average 8-year-old child weighing 25 kg, a dose of 37.2 μg/kg (*9*). This article provides information on a new area of research into EDs. It is extremely risky to extrapolate behavior in animals to behavior in humans. However, extensive human research about the relationship between EDs and aggressive behavior by young human males will be of interest.

In our study model, the mice sniffed intruders but did not attack, in contrast with other mouse studies (*4,8*). Whether or not the cumulative time of contact in our study is a good measure of aggression requires further discussion. Lagerspetz and Hautojarvi (*13*) evaluated the degree of aggression as follows: frequent nosing is more aggressive than no interest, and wrestling is more aggressive than frequent nosing. Palanza et al. (*4,8*) housed mice individually for 7 days to establish a home territory before an aggression test. This methodologic difference might be related to the more gentle character of our mice. Thus, the cumulative time of contact would seem to be a useful substitute for aggression in the present study. Testing might also have been better under red light illumination during the dark phase of the light cycle because mice are most active at night.

Decreased testis weight after early exposure. As for the effects on reproductive organs, treatment with bisphenol A at an early stage of life significantly decreased relative testis weight at 8 and 12 weeks of age compared with controls. The effects of relative testis weight of the low-dose group mice were temporary, as was the aggression score. vom Saal et al. (*12*) have reported that a 2-ng/g dose of bisphenol A decreased daily sperm production at 6 months of age. Recently, Cagen et al. (*20*) took a position against this result. They reported that a 2-ng/g dose of bisphenol A had no effects on male sexual development in mice (*20*), using the same experimental protocol as vom Saal et al. (*12*). In the present study, we did not examine this issue sufficiently, so we cannot say whether EDs had an effect on reproductive organ function. We can say that bisphenol A seems to have reduced relative testis weight temporally in young male mice. Both high- and low-dose bisphenol A groups showed increased aggressive behavior, yet the low-dose group actually had a smaller relative testis weight than did controls. Relative testis weight seems to have had no relationship to aggressive behavior in this model, nor did testosterone concentration.

Effect of low versus high dose on testis weight. It is interesting that a low dose (2 ng/g) of bisphenol A had an apparently larger effect on relative testis weight than did the much higher dose of 20 ng/g. A nonmonotonic, inverted-U dose response of DES on prostate size and the rate of territory marking in mice has been reported (*12,21*). vom Saal et al. (*12*) have suggested that unique outcomes may occur in response to low, environmentally relevant doses of EDs that will not be observed at higher doses. We were unable to clarify the mechanisms of the low-dose effects of EDs tested in this study. One possibility is that low-dose exposure to bisphenol A during the fetal period might result in the use of a different pathway that does not go through estrogen/androgen receptors toward the target organ.

In conclusion, our mouse model to evaluate aggression showed that male mice exposed to bisphenol A were more aggressive and had a reduction in relative testis weight compared with controls at 8 weeks of age.

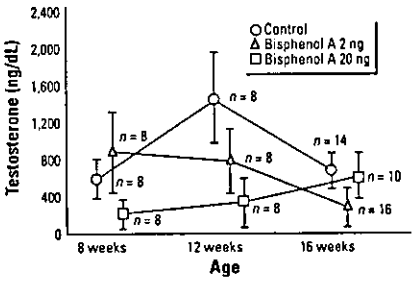

Figure 4. Chronologic change of serum testosterone concentration (mean ± SE) in control male mice and 2-ng/g bisphenol A and 20-ng/g bisphenol A male mice.

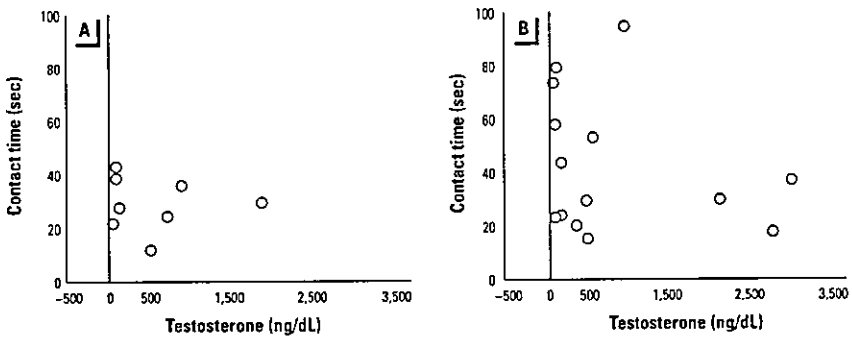

Figure 5. Correlation between contact time and testosterone concentration in 8-week-old male mice. (*A*) Control group ($r = 0.072$; $p = 0.82$). (*B*) Both treatment groups (2-ng/g bisphenol A and 20-ng/g bisphenol A) combined ($r = 0.237$; $p = 0.42$).

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