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Molecular cloning and histological localization of LH-like substances in a bottlenose dolphin (*Tursiops truncatus*) placenta

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Abstract

All mammals exhibit pituitary-specific expression of LH and FSH, whereas placental expression of gonadotropins has been reported only in primates and equids. Some cetaceans, such as dolphins, have a long gestational period and a sexual cycle of about 27 days almost comparable with that of humans. Histologically, dolphins have an epitheliochorial placentae that resembles placentas of Perissodactyla including horses. In the present study, we cloned cDNAs encoding gonadotropins and observed their immunohistochemical localization in the placenta of bottlenose dolphin. The cDNAs obtained encoded 120 amino acids for the α-subunit (including 96 amino acids of mature proteins), and 141 amino acids for the β-subunit (including 121 amino acids of mature proteins). The sequence of the α-subunit was similar to that in the pig (Artiodactyla) pituitary glycoprotein hormone [96.7% homology at amino acids (aa) level], and the sequence of the β-subunit was similar to that of luteinizing hormone (LH) in the pig [94.3% homology at aa level] and white rhinoceros (Perissodactyla) [93.3% homology at aa level]. Of interest, dolphin LHβ lacks carboxyl-terminal-peptides (CTP). This fact suggests that CTP are not essential for placental expression of gonadotropin in dolphins. Immunohistochemical observations employing anti-ovine LHβ antibody revealed positive staining in the villositycal tissue. Our observations suggest placental expression of gonadotropin homologues in cetaceans and possible evolutionary conservation of placentae-derived hormonal control of ovarian functions during pregnancy.

Keywords: Cetaceans; cDNA cloning; Chorionic gonadotropin; Luteinizing hormone; Immunohistochemistry; *Tursiops truncatus*; Placenta; Carboxyl-terminal-peptides

1. Introduction

The primary function of placentae is to exchange oxygen for carbon dioxide and nutrients for wastes between fetuses and mothers, and they also produce multiple hormones and bioactive substances. In viviparous mammals, progesterone, produced in the corpora lutea and placenta, is one of the key molecules responsible for maintaining a successful pregnancy. In rodents, gonadotropins produced in the pituitary gland have sole roles for maintenance of pregnancy, while in primate species, including humans, chorionic gonadotropin (CG) production from trophoblasts begins soon after implantation and stimulates ovarian progesterone production during early pregnancy. Whether of pituitary or placental origin, gonadotropins are evolutionarily conserved and share common molecular structures.

Human gonadotropins including luteinizing hormone (LH), follicle stimulating hormone (FSH), and chorionic gonadotropin (CG) belong to the glycoprotein hormone family and contain a common α-subunit and hormone-specific β-subunits. In various mammalian species, the DNA sequences of glycoprotein hormones were evolutionarily conserved while the placental expression of CG has thus far only been reported in primates and equids.

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Cetaceans, including dolphins, have long gestational periods (about 10–16 months) and sexual cycles of around 27 days, similar to those in primates, though their endocrine backgrounds of reproduction have not been well investigated. Hobson and Wide (1986) studied the bioactivity and immuno-reactivity of chorionic gonadotropin in animal placentae, including those of dolphins and humans, and found high levels of free CGβ activity in term dolphin placentae, in contrast to the high level of α-subunit activity in human placentae. Unfortunately, a complete molecular analysis was not conducted in their study.

Morphologically, dolphins have epitheliochorial placentae resembling horse placentae as well as those of cows and pigs. Though recent studies suggest linage relationships between Cetaceans and Artiodactyls (Ursing and Arnason, 1998, Gatesy et al., 1996), there are no published data suggesting placental expression of gonadotropin genes in 3 subgroups of Artiodactyls namely, Suiformes (pigs, peccaries, and hippopotamuses), Ruminantia (bovids, deer, tragilids and giraffes) and or Tylopoda (camels). Thus we performed molecular cloning of gonadotropins in a dolphin placenta as well as in a pituitary gland obtained from the same species.

2. Materials and methods

2.1. Tissue sampling

A placenta was obtained from a captive healthy female bottlenose dolphin (*Tursiops truncates*, named Azami, age unknown) after normal vaginal delivery on 11 Oct 2002 at Enoshima aquarium, Kanagawa prefecture. The placenta was incised into small pieces for morphological and molecular analyses. Frozen samples were kept at −80 °C for later RNA extraction.

The pituitary tissue was obtained from another wild female bottlenose dolphin captured in the traditional drive fisheries at Taiji-wan, Wakayama prefecture on 3 December 2003, which was delivered by the Wakayama Taiji fishery cooperative union. Tissue samples were obtained by craniotomy soon after euthanasic sacrifice by cutting spinal cord and were kept at −30 °C in RNA later® (TaKaRa Biotechnology, Shiga, Japan) storage solution for later use.

2.2. Amplification of cDNA encoding placental and pituitary gonadotropins

RNA was extracted from the tissue sampled by TRIzol® Regent (Gibco BRL, Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Single-strand cDNA was prepared using Ready-To-Go You-Prime® First-Strand Beads (Amersham Biosciences, Tokyo, Japan) using the vect-dT (Amersham Biosciences, Tokyo, Japan) adapter primer which were designed to anneal adapter sequences in the unknown 3′region, according to the manufacturer's instructions. To clone dolphin gonadotropins with unknown molecular homologies to those of other published animals, we used the degenerative PCR technique employing multiple primers. RT-PCR for synthesizing and amplifying of cDNA was carried out under the best conditions after several trials. Briefly, to amplify evolutionarily conserved sequences in the gonadotropin α-subunit genes, the primers were designed based on published α-subunit mRNA for rat LH (*Rattus norvegicus*, Godine et al., 1982), horse LH/CG (*Equus caballus*, Min et al., 1994), marmoset CG (*Callithrix jacchus*, Simula et al., 1995), and deer FSH (*Cervus nippon*, unpublished data).

For the β-subunit of LH, we referred to the published cDNA sequences for the giant panda (*Ailuropoda melanoleuca*, Liao et al., 2003), cow (*Bos taurus*; Maurer, 1985), white rhinoceros (*Ceratotherium simum*; Sherman et al., 1997), dog (*Canis familiaris*; Wolf et al., 1987), horse (*Equus caballus*; Sherman et al., 1992), donkey (*Equus asinus*; Chopineau et al., 1995), zebra (*Equus burchelli*; Chopineau et al., 1999), cat (*Felis catus*; unpublished data), and siberian tiger (*Panthera tigris altaica*; Crichton et al., 2003). The forward degenerate primers were designed after published mRNA sequences of mammalian hormones including the common α-subunit of CG, LH, and FSH, and the LH β-subunit. Adapter primers were designed to anneal adapter sequences in the unknown 3′region.

PCR was performed in an iCyclar™ (Bio-Rad Laboratories, Tokyo, Japan) in 2XGC buffer I supplemented with 5 units/μl TaKaRa LA Taq® polymerase, 2.5 mM of each dNTP Mixture (TaKaRa Biotechnology, Shiga, Japan), 10 pmol of degenerate primers, and 3.5 ng/2 μl of cDNA. The PCR condition was as follows: 35 cycle at 94 °C for 30 s, 55 °C for 30 s, and 72 °C 1 min 30 s. PCR products were separated by electrophoresis in 2% agarose gel and purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Woods Hollow Road Madison, WI, USA).

2.3. DNA sequencing and analysis

The amplified fragments were subcloned into pGEM-T vector using the pGEM-T vector Easy Vector System (Promega,

Table 1
Oligonucleotide primers used for PCR amplification of the glycoprotein cDNAs

Primer	Sequence
α-subunit	
alpha FW1 (A1)	5′ ATG GAT TAC TAC AGA ARA YAT GCA 3′
alpha FW2 (A2)	5′ CAT TCC YTT CCT GAT GGA GAG TTT A 3′
β-subunit	
beta FW1 (B1)	5′ ATG GAG ATG YTC CAG GGR CT 3′
beta FW2 (B2)	5′ ATC AAC GCC ACY CTG GCC GCT GAG AA 3′
adapter primer	
AP-1	5′ GTA ATA CGA CTC ACT ATA GGG C 3′
AP-2	5′ CTA TAG GGC ACG CGT GGT 3′

The forward common α-subunit primers (A1, A2) were designed based on the GenBank V01253: rat, GenBank AY066018: sika deer, GenBank U04446: common marmoset, and GenBank AB000200: horse glycoprotein hormone α-subunit cDNA sequences. The forward β-subunit primers (B1, B2) were designed based on GenBank AF448455: giant panda LHβ, GenBank M10077: bovine LHβ, GenBank U72659: white rhinoceros LHβ, GenBank Y00518: dog LHβ, GenBank S41704: horse LH/CGβ, GenBank X80116: donkey LHβ, GenBank Y16265: zebra LHβ, GenBank AF095716: cat LHβ, and GenBank AF354938: amur tiger LHβ cDNA sequences. AP were the adapter primers. R: A+G, Y: C+T.

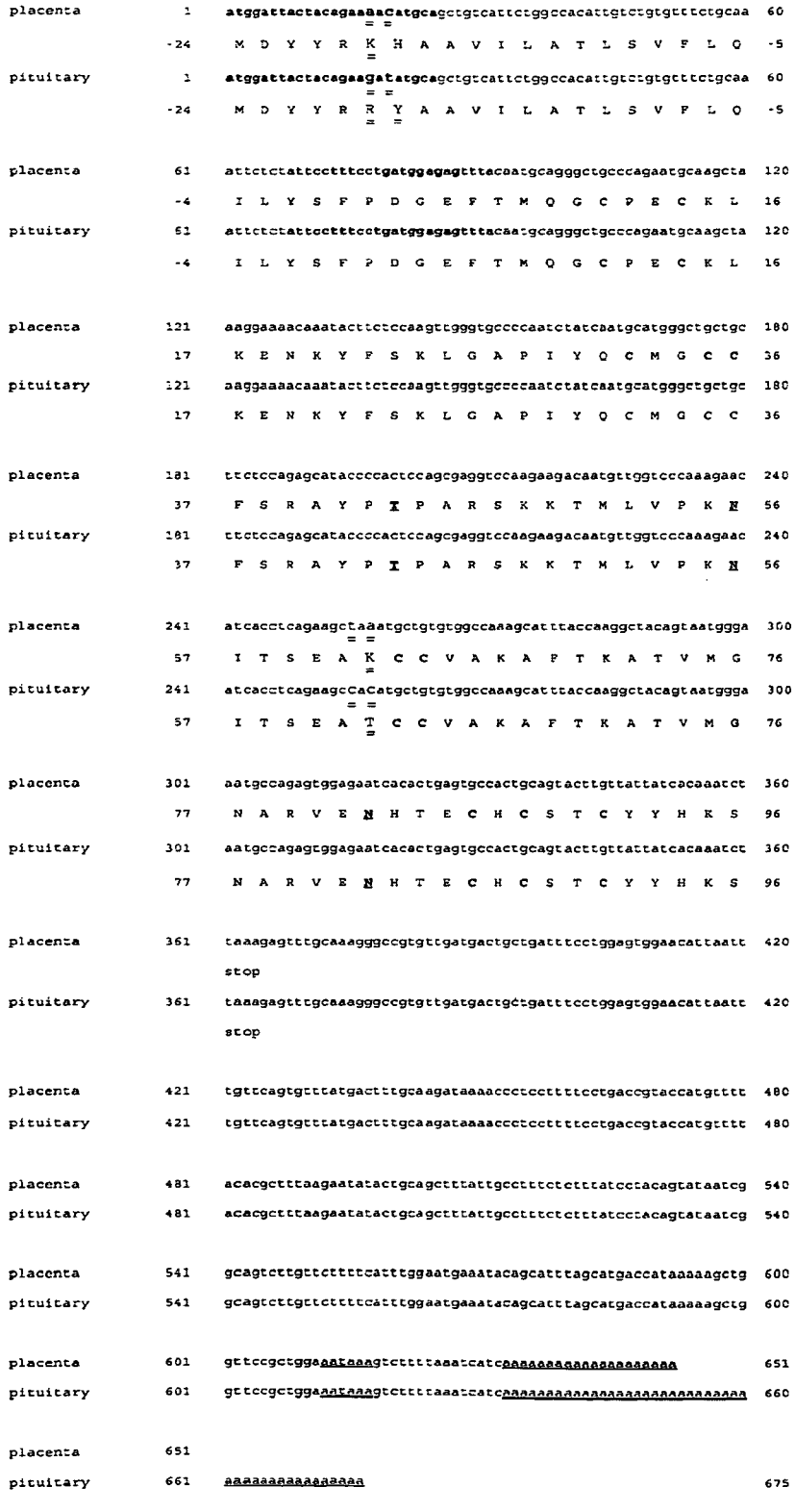

Fig. 1. cDNA nucleotide and deduced amino acid sequences of the bottlenose dolphin common glycoprotein α-subunit. The forward custom primers were designed from nucleotide positions 1 to 14(A1) and 68 to 91(A2). The primers sequences are shown in bold. The polyadenylation signal and poly(A)$^+$ tail are underlined. The free α-subunit is proposed to be the putative *O*-glycosylation site and the putative *N*-glycosylation sites are bolded and underlined. The Proposed proper subunit folding involves 10 cysteines, shown in bold. The nucleotide and amino acids residues showed a difference between the placenta and pituitary (double line).

Madison, USA) using the blunt-ended cloning method according to the manufacturer's manual. Vectors were transformed into competent cells (*E. coli* DH5α). Recombinant clones were selected using blue/white screening on X-gal/IPTG/ampicillin 2XYT plates. Then, positive clones for the α- and β-subunits were determined using PCR and enzymatic digestion with *Eco*RI. In order to avoid PCR and/or sequences errors, we tried sub-cloning with 2 or more bacterial colonies.

Positive clones were sequenced in both directions on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) using standard cycle conditions and the T7/SP6 promoter primer.

Nucleotide sequence data were compiled by using a computer software package: Sequence Editor Version 1.0.3 (Applied Biosystems, Tokyo, Japan). The sequence homology between the bottlenose dolphin and other published mammalians, obtained by the GenBank Database, were analyzed by the Blast (http://blast.genome.jp/) search program. Phylogenetic trees were calculated on the same program and displayed using Tree View version 1.5 software.

2.4. Immunohistochemistry

In order to localize expression of LH related cDNA in dolphin placentae, we examined placental tissues with immunohistochemical techniques. In the present study, we employed anti-ovine LHβ rabbit polyclonal antibody because the maximum homology was observed between ovine LHβ sequences (GenBank accession no. X52488) and newly sequences dolphin placenta derived LHβ cDNA. The placental tissue was fixed with Bouin fixation solution (15:5:1 of picric acid saturated solution in distilled water, 37% formaldehyde solution, glacial acetic acid) overnight. Then, after dehydration, the tissue was embedded with paraffin and cut into 5 μm sections on a microtome, which were then fixed onto slide glasses. Immunohistochemical staining was performed using LAB-SA Detection System (Histostain®-*Plus* Bulk Kit, ZYMED® Laboratories Inc., Invitrogen immunodetection, CA, USA) under the manufacturer's instruction with slight modifications. Briefly, deparaffinized sections were subjected to an antigen-retrieval step in TUF™ Target Unmasking Fluid (MONOSAN, Uden, The Netherlands) at 90 °C for 10 min. The sections were incubated with 0.6% H_2O_2 solution to inhibit endogenous peroxidase activity, and then washed in distilled water for 10 min. The sections were blocked with a solution of normal goat serum. After draining the blocking serum, the sections were incubated with the primary antibody [Rabbit Anti-ovine LH β subunit serum: code name HAC-OV27(β)-01RBP85 (Laboratory of Biosignal Sciences Inst. for Molecular and Cellular Regulation, Gunma University)], diluted to 1:500 in phosphate-buffered saline (PBS) at 4 °C overnight after standing at room temperature for 30 min. After three washes in distilled water and through PBS, the slides were incubated with biotinylated anti-

Table 2
GenBank accession numbers employed as the sources of vertebrate glycoprotein hormones

(Class) Order	Common name	Species	Genbank accession no.		
			α-subunit	β-subunit	
				LHβ	CGβ
(Mammalia)					
Marsupialia	Brush-tailed possum	*Trichosurus vulpecula*	AF017447	AF017448	
	Red kangaroo	*Macropus rufus*	AF017449	AF017450	
Cetartiodactyla	Pig	*Sus scrofa*	D00768	D00579	
	Bovine	*Bos taurus*	X00050	M10077	
	Sheep	*Ovis aries*	X16977	X52488	
Perissodactyla	Donkey	*Equus asinus*	X85170	X80116	
	Plains zebra	*Equus burchelli*	Y16326	Y16265	
	Horse	*Equus caballus*	AB000200	S41704 (LH/CHβ)	
	White rhinoceros	*Ceratotherium simun*	nonpublished	U72659	
Carnivora	Dog	*Canis familiaris*	AF160250	Y00518	
	Cat	*Felis silvestris catus*	AY972823	AF095716	
Rodentia	Mouse	*Mus musculus*	J00643	Y10418	
	Golden hamster	*Mesocricetus auratus*	AF307148	AY353074	
Primates	Human	*Homo sapiens*	V00518	X00264	J00117
	Common marmoset	*Callithrix jacchus*	U04446	nonpublished	U04447
	Crab eating macaque	*Macaca fascicularis*	AY026358	AJ781396	Y026359
(Aves)					
Galliformes	Common turkey	*Meleagris gallopavo*	M33698	L35519	
	Common quail	*Coturnix coturnix*	S70833	S70834	
(Osteichthyes)					
Anguilliformes	European eel	*Anquilla anquilla*	X61038	X61039	
Siluriformes	African catfish	*Clarias gariepinus*	X97760	X97761	
Salmoniformes	Chum salmon	*Oncorhynchus keta*	M27653	M27154	

The common α-subunit, LHβ, and CGβ sequences were used for amino acid alignment sequencing and phylogenetic analysis in the present study.

rabbit secondary antibody (included in the detection kit) at room temperature for 30 min. Then, the sections were washed three times in distilled water and treated with the streptavidin-HRP solution at room temperature for 15 min. After three washes in distilled water and through PBS, the sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, WAKO, Osaka, Japan) solution for 2 min.

3. Results

3.1. Cloning of the gonadotropin common α-subunit cDNA

We amplified one major fragment from the dolphin placenta and pituitary cDNA library by the first PCR using the A1 and AP-1 primers, followed by nested PCR using the A2 and AP-2 primers (Table 1). We obtained 651 and 675 base pair (bp) fragments as candidates for the putative dolphin gonadotropin α-subunit (Fig. 1). The amino acid sequence deduced from these fragments showed higher homologies to mammalian glycoprotein hormones (Table 3). The end of the signal peptide and the beginning of the mature protein amino acid sequence was designated as the −1/+1 boundary (Table 2). When a signal sequence of 24 amino acids was located, the proposed mature α-subunit of dolphin gonadotropin started with cysteine and consisted of 96 amino acids. The free α subunit is proposed to be *O*-glycosylated at Thr-43. A putative *N*-linked glycosylation site was located at Asn-56 and Asn-82 from the N-terminus of the predicted mature peptide. Fig. 2 shows the alignment of amino acid sequences of the dolphin gonadotropin α-subunit and those of other mammals. All of the 10 cysteines (form 5 disulfide bonds), the putative *O*-linked glycosylation site, and the putative two *N*-linked glycosylation sites of the dolphin gonadotropin α-subunit were completely conserved among the mammals.

We cloned gonadotropin α-subunit-like sequences from cDNA libraries prepared from placental and pituitary tissues, and observed nucleotide substitutions in 17 (A/G), 19 (C/T), 255 (T/C), and 257 (A/C). The deduced amino acid sequences differed between the placenta and pituitary gland, as follows: AA −19 (Lys−>Arg),−18 (His−>Tyr), and 62 (Lys−>Thr).

3.2. Cloning of the gonadotropin β-subunit cDNA

We obtained 539 and 534 bp fragments as candidates for the putative dolphin gonadotropin β-subunit (Fig. 3), which we amplified from the dolphin placenta and pituitary cDNA library using B1, B2, AP-1, and AP-2 primers (Table 1). Surprisingly, the deduced amino acid sequence from these fragments showed higher homology to mammalian LHβ genes than to CGβ genes cloned in primates or horses (Tables 3 and 4). By comparing the sequence with that of other mammalian LHβ subunits, a signal peptide of 20 amino acids and a mature peptide of 121 amino acids were predicted for the dolphin gonadotropin β-subunit (Fig. 4). The end of the signal peptide and the beginning of the mature protein amino acid sequence were designated as the −1/+1 boundary. A putative *N*-linked glycosylation site was found at

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            -24        -10        1          10         20         30         40         50         60         70         80         90    96
                                               * *                   *  **                            **                          *   *   *
                                                                                   ⇩                     ⇩                          ⇩
placenta    MDYYRKHAAV ILATLSVFLQ ILYSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEAKCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
pituitary   MDYYRRYAAV ILATLSVFLQ ILYSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
possum      MDYYRKYAAV ILATLSVFLH ILHSFPDGEF IMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYYHKS
kangaroo    MDYYRKYAAV ILATLSVFLH ILHSFPDGEF IMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYYHKS
pig         MDYYRKYAAV ILAILSVFLQ ILHSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
bovine      MDYYRKYAAV ILTILSLFLQ ILHSFPDGEF TMQGCPECKL KENKYFSKPD AAIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NVRVENHTEC HCSTCYYHKS
sheep       MDYYRKYAAA ILAILSLFLQ ILHSFPDGEF TMQGCPECKL KENKYFSKPD APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NVRVENHTEC HCSTCYYHKS
dog         MDCYRKYAAV ILAALSVFLH ILHSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYYHKS
cat         MDYYRKYAAV ILAILSVFLH ILHSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYHHKI
mouse       MDYYRKYAAV ILVMLSMFLH ILHSLPDGDF IIQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
hamster     MDYYRKYAAV ILVVLSMFLH ILHSLPDGDF MIQGCPECKL KENKYFSKLG APIYQCMGCC FSSAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
donkey      MDYYRKHAAV ILATLSVFLH ILHSFPDGEF TTQDCPECKL KKNKYFSKLG VPIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFIRVTLMG NIRLENHTQC YCSTCYHHKI
zebra       MDYYRKHAAV ILATLSVFLH ILHSFPDGEF TTQDCPECKL KVNKYFSKLG VPIYQCMGCC FSRAYPTPAR SRKTMLVPKN ITSEATCCVA KAFIRVTLMG NIRLENHTQC YCSTCYHHKI
horse       MDYYRKHAAV ILATLSVFLH ILHSFPDGEF TTQDCPECKL RENKYFFKLG VPIYQCKGCC FSRAYPTPAR SRKTMLVPKN ITSESTCCVA KAFIRVTVMG NIKLENHTQC YCSTCYHHKI
human       MDYYRKYAAI FLVTLSVFLH VLHSAPD--- -VQDCPECTL QENPFFSQPG APILQCMGCC FSRAYPTPLR SKKTMLVQKN VTSESTCCVA KSYNRVTVMG GFKVENHTAC HCSTCYYHKS
marmoset    MDYYRKYAAI ILITLSVFLH ILHSLPDGEF TAEECPECKL KENKYFSRLG SPIYQCMGCC FSRAYPTPLR SQKTMLVPKN VTSESTCCVA KAYTKATVMG NIRVENHTEC HCSTCYHHKF
macaque     MDYYRKYAAV ILVTLSVFLH ILHSFPDGEF TMQDCPECKP RENKFFSKPG APIYQCMGCC FSRAYPTPLR SKKTMLVQKN VTSESTCCVA KSLTRVMVMG NVRVENHTQC HCSTCYYHKF
turkey      MDCYRKYAAV TLTILSVFLH LLHTFPDGEF LMQGCPECKL GENRFFSKPG APIYQCTGCC FSRAYPTPMR SKKTMLVPKN ITSEATCCVA KAFTKITLKD NVKIENHTDC HCSTCYYHKS
quail       MDCYRKYAAV TLTILSVFLH LLHTFPDGEF LMQGCPECKL GENRFFSKPG APIYQCTGCC FSRAYPTPMR SKKTMLVPKN ITSEATCCVA KAFTKITLKD NVKIENHTDC HCSTCYYHKS
e.eel       MMVCPGKPGA SLLMLSMLFH IIDSYPNNEM ARGGCDECRL QENKIFSKPS APIFQCVGCC FSRAYPTPLR SKKTMLVPKN ITSEATCCVA REVTRL---D NMKLENHTDC HCSTCYYHKF
a.catfish   MTLIPKYTGA TILLLCVLIE IGQLYPNNDF ---GCEECKL KENNIFSKPG APVYQCMGCC FSRAYPTPLR SKKTMLVPKN ITSEATCCVA KEVKRVIVND -VKLVNHTDC HCSTCYYHKF
salmon      ---------- -SLILSILLY MADSYQNSDM TNVGCEECKL KENKVFSNPG APVYQCTGCC FSRAYPTPLQ SKKAMLVPKN ITSEATCCVA KEGERVVV-D NIKLTNHTEC WCNTCYHHKS
                                   *.:::  :     :     * **      *   *  .  .  .: ** *** ** ***** : *:*:*** ** :***:.**** :   :          :: *** *   *.***:**
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Fig. 2. Multiple sequence alignment of the glycoprotein hormone common α-subunit. The deduced amino acid sequences of the bottlenose dolphin glycoprotein hormone common α-subunit gene are aligned with these vertebrates. Gaps (−) are inserted to obtain maximum homology. Amino acid positions are indicated on the top. Ten conserved cysteines are denoted by "*", two putative *N*-linked glycosylation sites, and one *O*-linked glycosylation site by "⇩". Conserved and similarity residues are indicated by "*", "." or ":" under sequences.

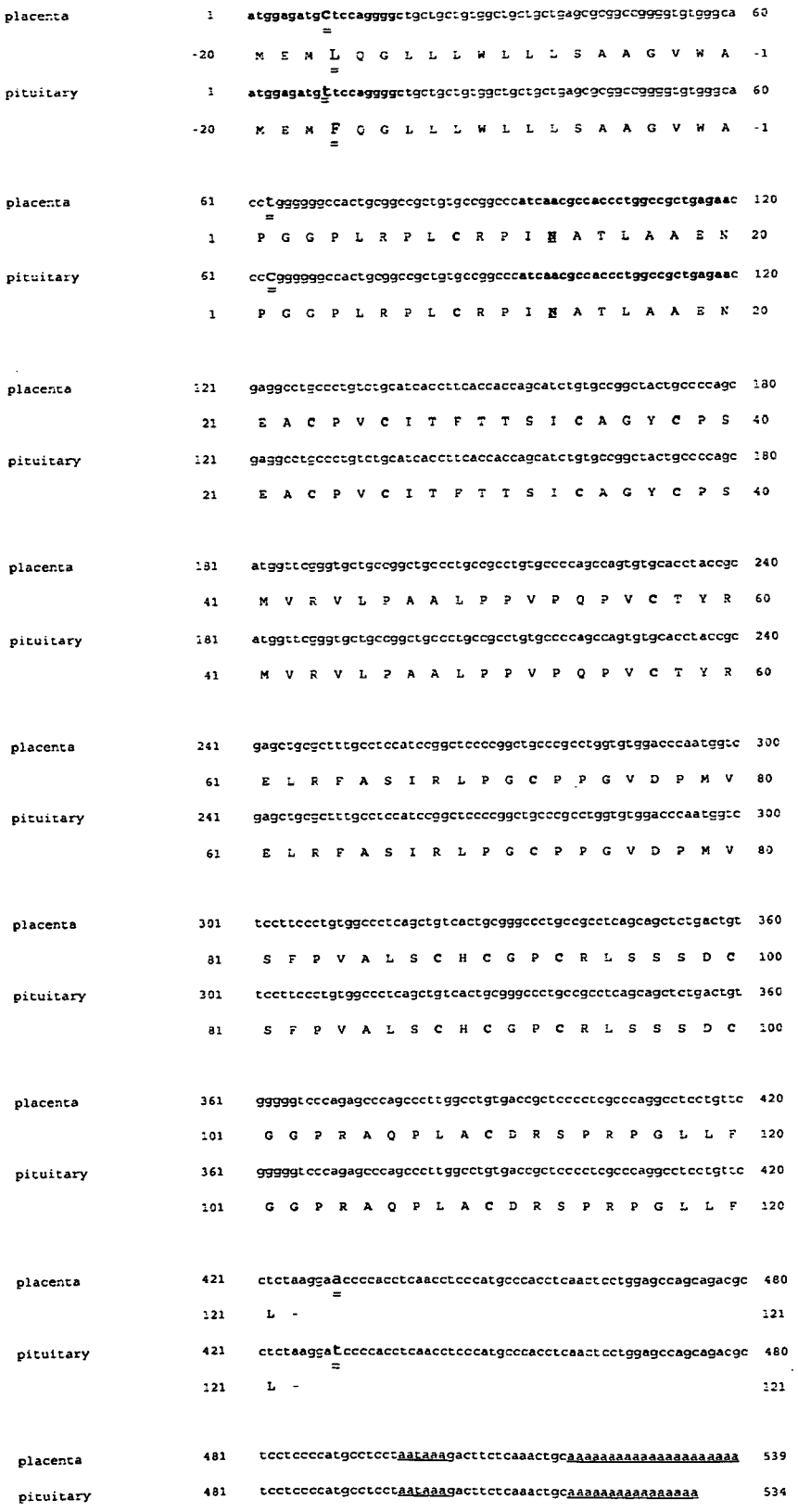

Fig. 3. cDNA nucleotide and deduced amino acid sequences of the bottlenose dolphin gonadotropin β-subunit. The forward custom primers were designed from nucleotide positions 1 to 20(B1) and 94 to 119(B2). The primers sequences are shown in bold. The polyadenylation signal and poly(A)$^+$ tail are underlined. The *N*-glycosylation sites are shown in bold and are underlined. The proposed proper folding involves 12 cysteines shown in bold. The nucleotide and amino acids residues showed that there was a difference between the placenta and pituitary (double line).

Table 3
Nucleotide similarities between the bottlenose dolphin placental cDNAs and those of other vertebrates

Common name	Bottlenose dolphin gonadotropin α-subunit cDNA Homology (%)	Bottlenose dolphin gonadotropin β-subunit cDNA Homology (%)
Pituitary	99.4	99.4
Brush-tailed possum	75.8	68.3
Red kangaroo	76.6	69.9
Pig	NA	NA
Bovine	89.4	85.4
Sheep	89.6	88.0
Donkey	87.6	85.0
Plains zebra	87.3	86.1
Horse	85.4	68.8
White rhinoceros	NA	91.0
Dog	93.3	87.5
Cat	93.4	89.6
Mouse	73.3	85.8
Golden hamster	72.7	80.8
Human	83.1	LH: 67.8 CG: 77.4
Common marmoset	86.0	CG: 78.9
Crab eating macaque	87.9	LH: 84.5 CG: 81.8
Common turkey	66.6	52.8
Common quail	66.5	53.9
European eel	58.1	53.3
African catfish	58.7	58.1
Chum salmon	56.2	60.3

Similarities were calculated as the percent of identical nucleotides over the entire coding regions. Note: NA, cDNA data are not available.

Asn-13 from the N-terminus of the putative mature peptide. All of the 12 cysteines and the putative *N*-linked glycosylated site of the dolphin gonadotropin β-subunit were completely conserved among the mammals (Fig. 5). A phylogenic analysis showed that the dolphin placental and pituitary gonadotropin β-subunits belong to the cluster of mammalian LHβ genes (Fig. 6).

In the present study, we cloned LHβ-like sequences from cDNA libraries prepared with placental and pituitary tissues. As with LHβ, we observed two nucleotide substitutions, 9(C/T) and 63(T/C), in the coding region and a single substitution, 430 (A/T), in the untranslated region. The deduced amino acid sequence differed between the placenta and pituitary gland as follows: AA-17 (Leu−>Phe) (Table 4).

3.3. Statistical analysis

We tried PCR based cloning with at least 2–3 colonies. For placenta LHα, 3 of 3 colonies showed identical cDNA sequences. For placenta LHβ, 2 of 2 colonies showed identical cDNA sequences.

In pituitary LHα, 3 of 3 colonies showed identical cDNA sequences, while in pituitary LHβ the 1st and 2nd subclones showed 2 base pair differences. Then we tried to sequence 3 additional colonies and obtained identical cDNA sequences with the 1st one. Thus, we considered the difference was arisen from PCR or sequencing errors.

3.4. Immunohistochemistry

To determine the localization of the LH β-like subunit, we performed enzyme labeled immunohistochemistry on sections of a dolphin placenta. Anti-ovine LH β-subunit anti-sera, applied to a dolphin placenta, stained the outer syncytiotrophoblasts. No labeling was seen in the interior layer of cytotrophoblasts or fibrovascular connective tissue cores (Fig. 7−1,4). We observed negative staining replaced primary antibody with PBS (Fig. 7−3) and kidney (Fig. 7−6) and other tissues(data not shown).

4. Discussion

Glycoprotein hormones, such as follicle-stimulating hormones (FSHs), luteinizing hormones (LHs), thyroid-stimulating hormones (TSHs), and chorionic gonadotropin (CG), are key endocrine hormones secreted from the pituitary gonadotrophs and thyrotrophs and the placenta in primates. In primates, successful pregnancy depends on placental CG expression. CG is a glycoprotein hormone expressed in the human placenta that binds to LH/CG receptors on the corpus luteum and prevents its regression at menstruation. It also stimulates continued progesterone production which maintains the uterine lining in a specialized state that is receptive to implantation and placental development, and which regulates maternal immune responses. However, CG has not been found in other mammalian orders. Recent genomic analyses have shown that CGβ genes do not exist

Table 4
Deduced amino acid sequence similarities between bottlenose dolphin placental gonadotropins and other gonadotropins of other vertebrates

Common name	Bottlenose dolphin gonadotropin α-subunit a.a. Homology (%)	Bottlenose dolphin gonadotropin β-subunit a.a. Homology (%)
Pituitary	97.5	99.3
Brush-tailed possum	95.0	73.4
Red kangaroo	95.0	75.4
Pig	96.7	94.3
Bovine	91.7	84.2
Sheep	92.5	84.4
Donkey	86.6	83.2
Plains zebra	85.7	84.7
Horse	83.2	81
White rhinoceros	NA	93.3
Dog	94.2	90.6
Cat	94.1	88.1
Mouse	90.8	96.5
Golden hamster	90.0	85.8
Human	70.8	LH: 67.9 CG: 72.3
Common marmoset	84.0	CG: 71.8
Crab eating macaque	82.4	LH: 75.9 CG: 77.9
Common turkey	79.2	46.4
Common quail	79.2	48.2
European eel	62.5	44.3
African catfish	60.5	42.1
Chum salmon	59.1	42.3

Similarities are expressed as the percent of identical amino acids over the total for the species.

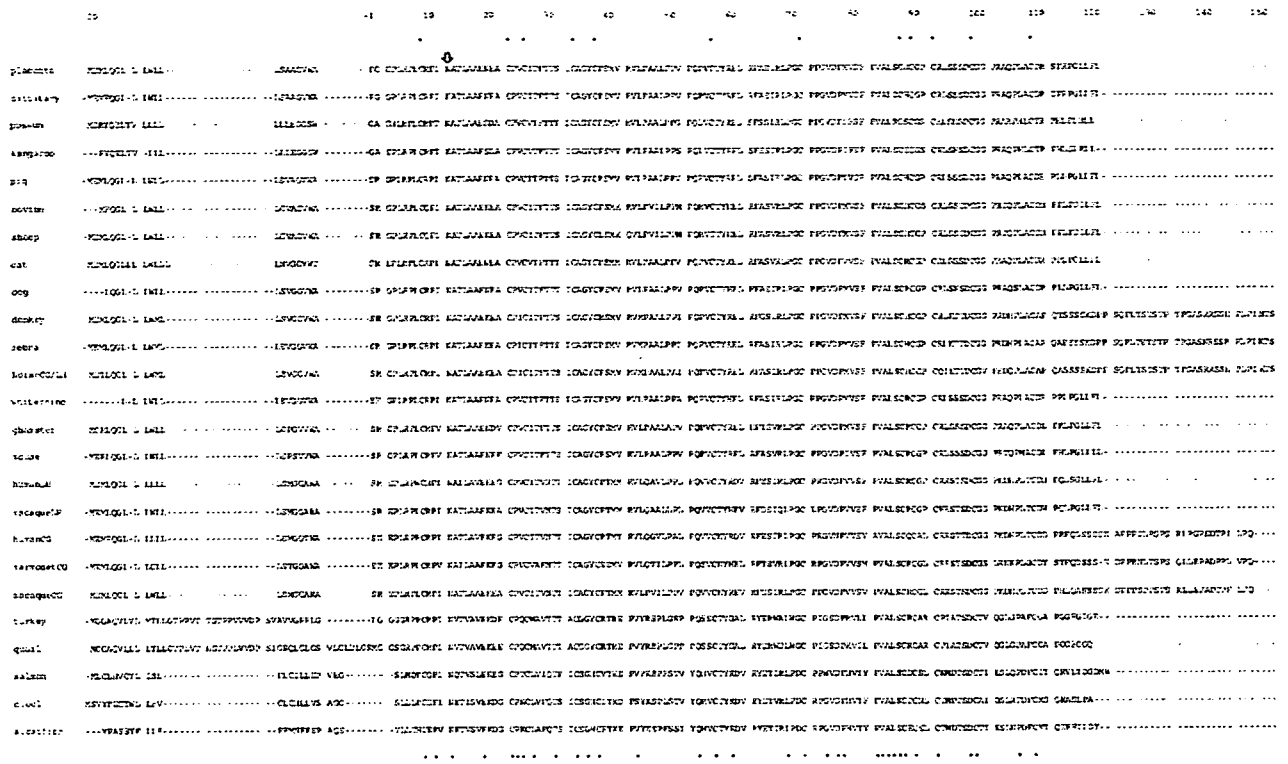

Fig. 4. Multiple sequence alignment of the gonadotropin β-subunit. The deduced amino acid sequences of the bottlenose dolphin gonadotropin β-subunit gene are aligned with these vertebrates. Gaps (−) are inserted to obtain maximum homology. Amino acid positions are indicated on the top. Residues identical to bottlenose dolphin gonadotropin β-subunit are presented in white letters. Twelve conserved cysteines are denoted by "*", and a putative *N*-linked glycosylation site by "⇓". Conserved residues are indicated by "*" under the sequences.

in rats (Jameson et al., 1984; Tepper and Roberts, 1984; Carr and Chin, 1985), mice (Kumar and Matzuk, 1995), cows (Virgin et al., 1985), pigs (Ezashi et al., 1990), sheep (Brown et al., 1993), or the rhinoceros (Lund and Sherman, 1998). Though there is only one report, employing highly sensitive RT-PCR technique, suggesting mRNA expression of LH in rat placentae (Shinozaki et al., 1997), most researchers regard placental expression of gonadotropin is observed only in primates and equids. From comparative molecular analysis of cDNA structure, Maston and Ruvolo (2002) proposed its recent origin within primates. It is noteworthy that horses have evolved placental LH expression, which is functionally convergent upon the anthropoid CG, but with a different molecular basis (Sherman et al., 1992).

In the present study, we cloned LHβ-like substances in a dolphin placenta. To our knowledge, this is the second report to show evolutionary convergence of glycoprotein hormones among mammalian placentae. However, it is improbable that only horses and dolphins express LHβ in their placentae. The placentae from other mammalian animals, viviparous reptiles, and fishes are possible candidates for the expression glycoprotein hormones, since a recent article reported evolutionary conservation of glycoprotein hormones, with α- and β-subunits, not only in vertebrates but also in invertebrate animals (Hsu et al., 2002; Sudo et al., 2005; Park et al., 2005). LH and FSH genes were cloned in all classes of vertebrates including pisces (Chatterjee et al., 2005; Degani et al., 2003; Gen et al., 2000; Hassin et al., 1995; Hsieh et al., 2001; Hellqvist et al., 2004; Huggard-Nelson et al., 2002; Hurvitz et al., 2005; Jackson et al., 1999; Kim et al., 2005; Kitahara et al., 1988; Kumar and Trant 2004; Koide et al., 1992; Kwok et al., 2005; Li et al., 2005; Mateos et al., 2003; Parhar et al., 2003; Querat et al., 1990a,b, 2004; Rebers et al., 1997; Sekine et al., 1989; So et al., 2005; Vischer and Bogerd, 2003; Weltzien et al., 2003), amphibians (Komoike and Ishii, 2003; Saito et al., 2002), Reptiles (Aizawa and Ishii, 2003; Chien et al., 2005), birds (Ando and Ishii, 1994; Foster and Foster, 1991; Kawasaki et al., 2003; Kikuchi et al., 1998; You et al., 1995) as well as mammals (Bello et al., 1989; Brown et al., 1993; Chin et al., 1981, 1983; Chopineau and Stewart, 1996; Chopineau et al., 1999; Crawford et al., 1986; Crichton et al., 2003; D'Angelo-Bernard et al., 1990; Degani et al., 2003; Ezashi et al., 1990; Fiddes and Goodman, 1979, 1980; Fiddes and Talmadge, 1984; Godine et al., 1982; Harrison et al., 1998; Hirai et al., 1989; Jameson et al., 1984; Kato and Hirai, 1989; Kato et al., 1991; Koura et al., 2004; Liao et al., 2003; Lund and Sherman, 1998; Lovejoy et al., 1992; Maurer, 1985; Nilson et al., 1983; Schmidt et al., 1999; Sherman et al., 2001, 1997, 1992; Simula, 1995; Tepper and Roberts, 1984; Virgin et al., 1985; Wolf et al., 1987; Yang et al., 2000; Zanella et al., 1996). Thus we can expect to find expression of LH or related glycoprotein hormones in placenta other than primates, equids and dolphins.

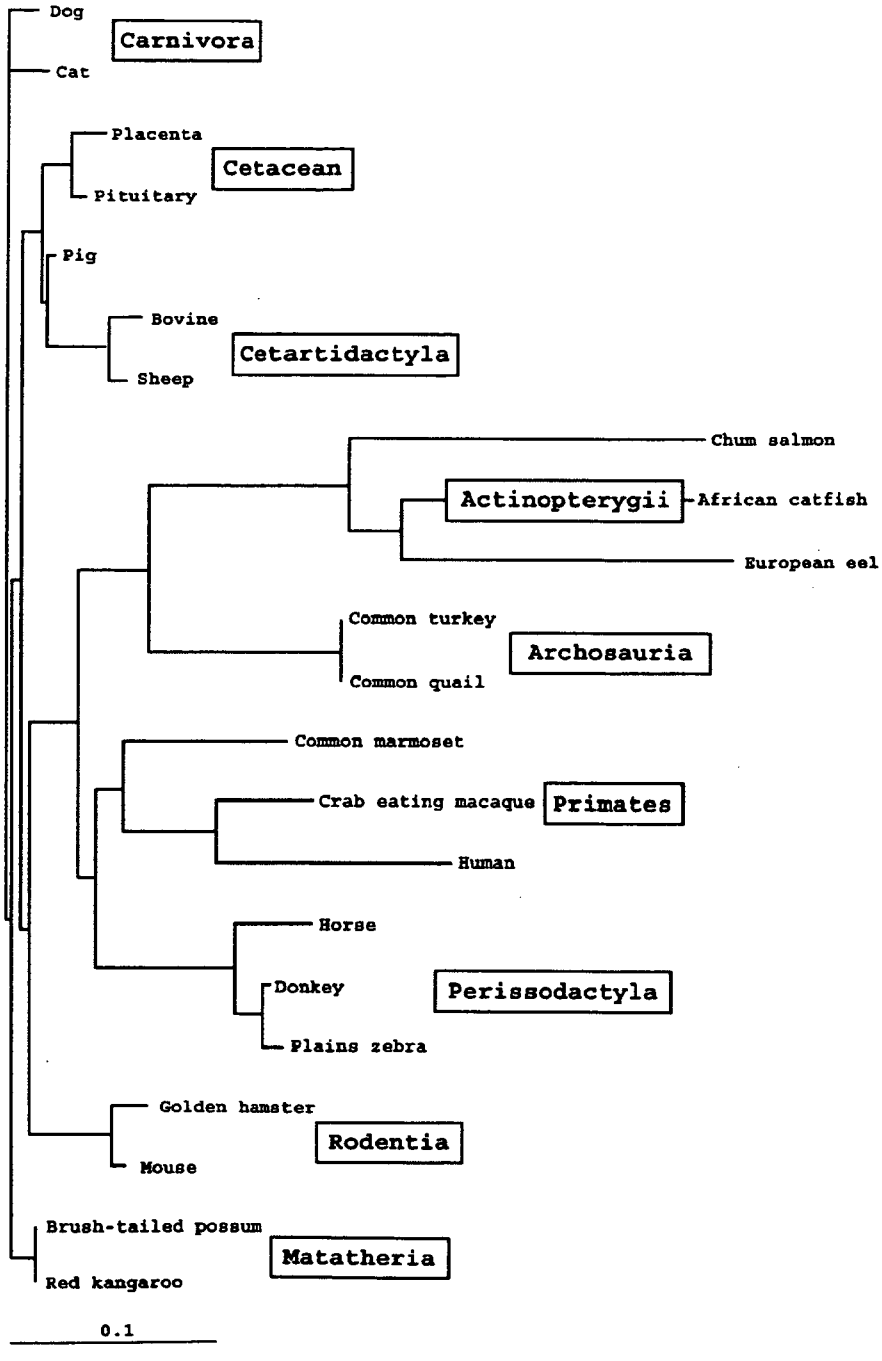

Fig. 5. Phylogenetic tree of the amino acid sequences of the vertebrate gonadotropin common α-subunits. The lengths of the horizontal lines indicate genetic distances. The sequences were made with reference to the same studies cited in Figs. 1 and 2.

Because one of the authors recently presented a hypothesis that viviparity is evolved from its evolutionary advantage on reproductive success in all vertebrate classes except for Avians (Hayakawa, 2006).

Of interest, we cloned the α-subunit (common to pituitary glycoprotein hormones) and LH β-subunit-like sequences from a dolphin placenta and found that the cDNA sequences showed several point mutations from pituitary cDNA. We propose two possible explanations. First, dolphins may have two or more genes coding the common α-subunit and LH β-subunits. In fact, primate genomes contain at least two LHβ analogues (LHβ and CGβ). However, we consider this explanation to be improbable because most mammalian species have only single common α-subunit gene and molecular homologies between dolphin placenta derived LHβ and pituitary LHβ and because the placenta-derived α-

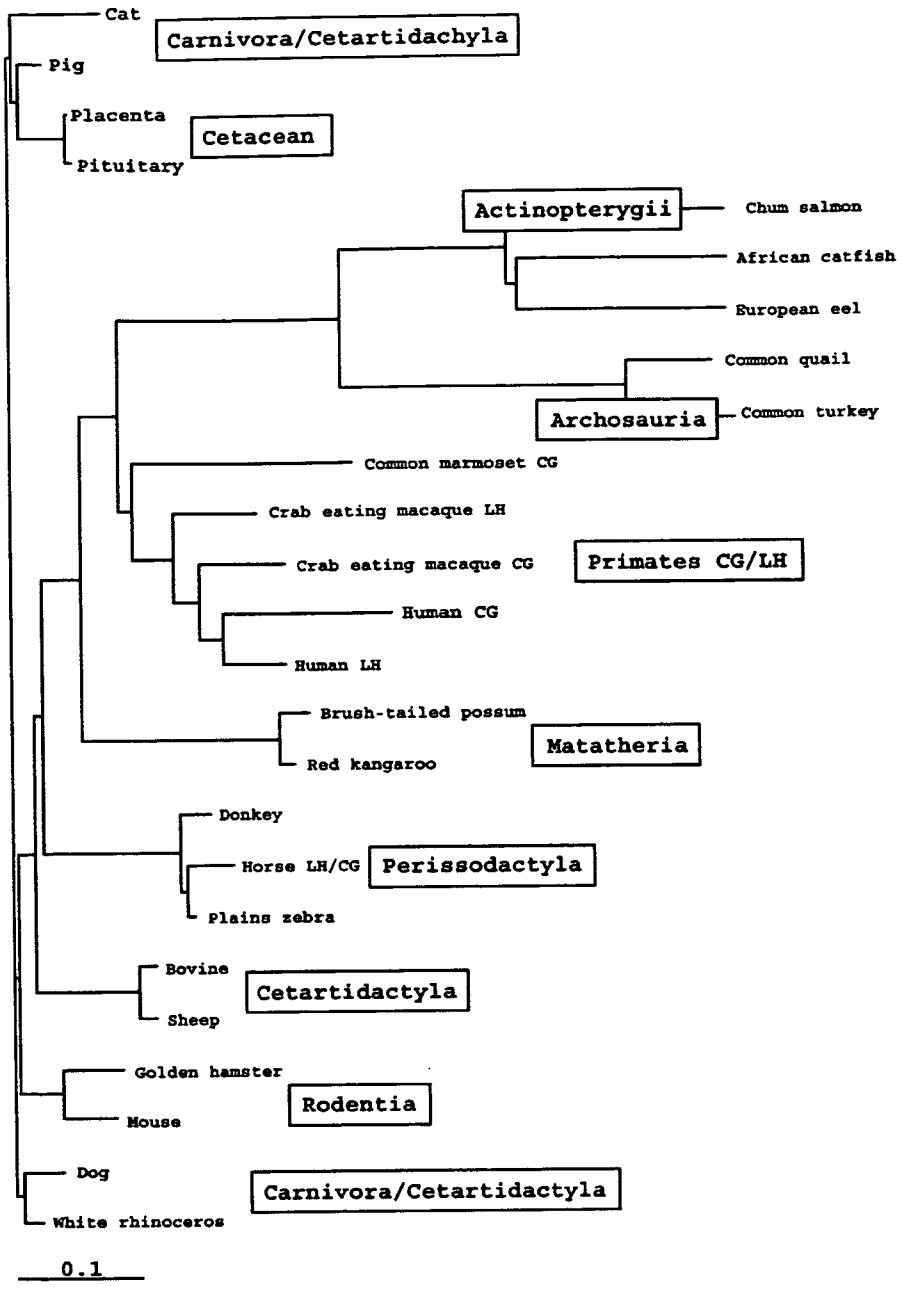

Fig. 6. Phylogenetic tree of the amino acid sequences of the vertebrate gonadotropin β-subunits. Genetic distances are indicated by the lengths of the horizontal lines. The sequences were made with reference to the same studies cited in Figs. 3 and 4.

subunit and pituitary α-subunit were highly homologous compared with hCGβ and human LHβ.

Second, these genetic differences were derived from a single nucleotide polymorphism (SNP) in bottlenose dolphins. Although most scientists today only recognize one species of bottlenose dolphin, the variations have been classified into at least three subspecies. Some physical characteristics vary so much among them that separate species classifications have been suggested. The captive Azami in Enoshima aquarium and the wild dolphin captured at Taiji may have had different genetic backgrounds. Due to the relative inaccessibility of wild dolphin placentae, it is difficult to establish cDNA cloning of LH genes both from placental and pituitary tissues simultaneously.

Recently, carboxyl terminal peptide (CTP) sequences of the chorionic gonadotropin (CG) beta subunit have been cloned in primates and horses genes. These sequences are considered to serve as an effective linker to enhance the secretion of the analogs compared to variants lacking the CTP (Nakav et al., 2006). However, as the gonadotropin

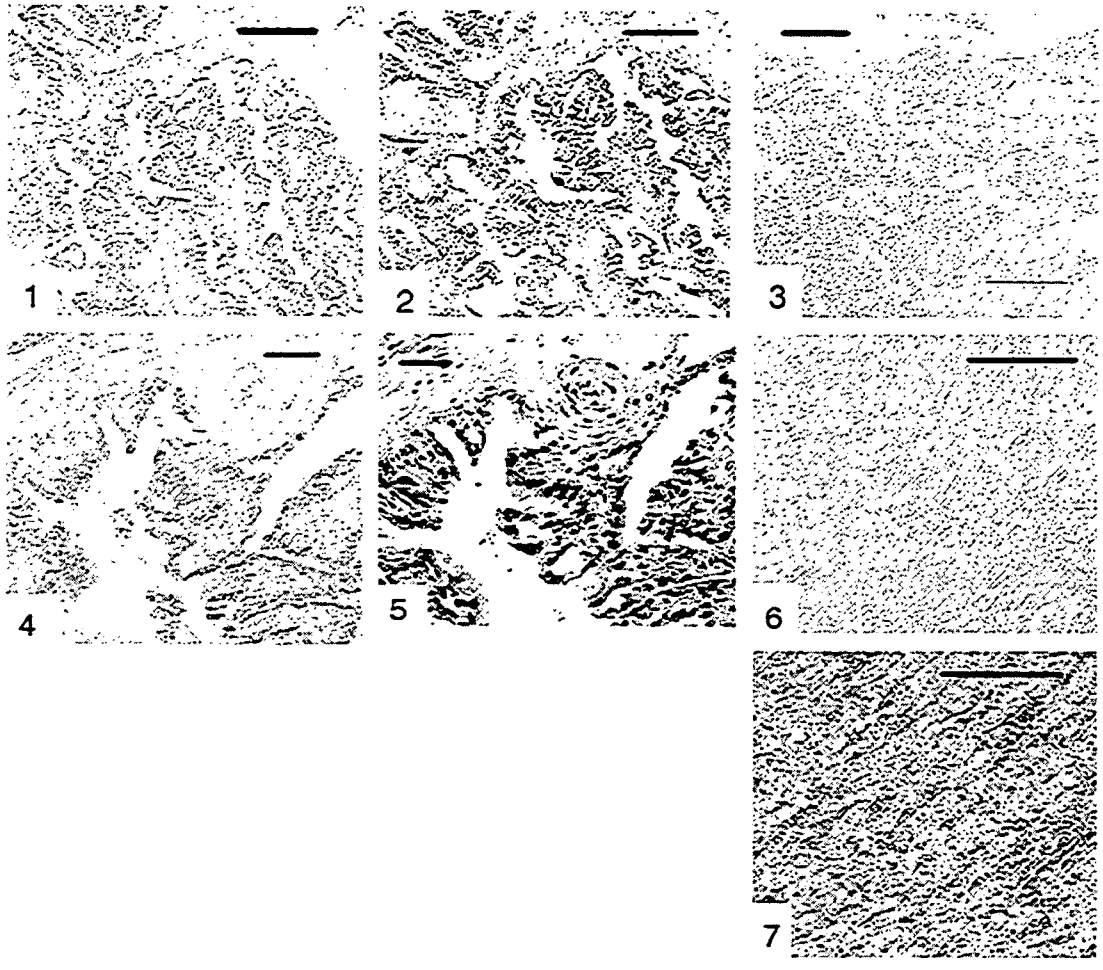

Fig. 7. Immunohistochemical distribution of the LHβ-subunit-like substance(s) in a dolphin placenta. 1, Low-power image of dolphin placenta showing the immunohistochemical staining treated with rabbit anti-ovine LH β-subunit serum. 2, Low-power image of hematoxyline/eosine staining of dolphin placenta. 3, The negative control set up by omitting the primary antibody. 4, High-power image of dolphin placenta showing the immunohistochemical staining treated with rabbit anti-ovine LH β-subunit serum. 5, High-power image of hematoxyline/eosine staining of dolphin placenta. 6, The negative control set up by kidney of dolphin. 7, Image of hematoxyline/eosine staining of dolphin kidney. Bars: 200 μm (1,2,3,6,7), 30 μm (4,5).

subunits of most non-primates, non-equid species lack a CTP domain suggesting it is not essential for endocrine function. Further, primate and equine CTP sequences were generated by read-through of consensus translation stop codons and frame-shift mutation (Fiddes and Talmadge, 1984; Sherman et al., 1992). In the present study, we searched CTP and/or related sequences in cDNA obtained from dolphin placenta and pituitary samples but failed to find out these sequences.

We identified the stop codon in 3′ terminus of LHβ genes in cDNA clones obtained from both placenta and pituitary gland. Taken together, though CTP is effective for endocrine functions, it is not essential for gonadotropin functions at least in dolphins. In other words, the primordial mammalian LHβ gene existed in the common ancestor of Perissodactyla, Artioductyls (putative ancestor of dolphins) and primates. CTP sequences evolved toties quoties by different genetic mechanisms by its virtue of endocrine efficiency.

In conclusion, we identified placental expression of LH-like substance(s) in a bottlenose dolphin for the first time in non-primate animals other than equids. Our findings suggest the evolutionary convergence of placenta-derived hormone(s) for the maintenance of pregnancy. Further studies are requested to better understand the genetic evolution of glycoprotein families in Cetaceans.

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ORIGINAL ARTICLE

Expression of Inducible Microsomal Prostaglandin E Synthase in Local Lesions of Endometriosis Patients

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Problem
Recently, an inducible microsomal human prostaglandin E synthase (mPGES) was identified. This enzyme converts the cyclooxygenase (COX) product, prostaglandin (PG) H_2, to PGE_2, an eicosanoid linked to carcinogenesis. Although elevated levels of PGE_2 have been observed in many tumor types including colorectal adenomas and cancers, its role in the pathophysiology of endometriosis is unknown. We previously reported increased expression of COX-2 messenger RNA (mRNA) in local lesions of endometriosis. To further elucidate the mechanism responsible for the elevated levels of PGE_2 in endometriosis, we examined the expression levels of mPGES.

Method of study
Samples were obtained from 28 patients, fixed in formalin, and embedded in paraffin for immunohistochemical analysis. We examined the expression of mPGES mRNA in seven cases by reverse transcriptase-polymerase chain reaction using total RNA extracted from frozen samples.

Results
Immunohistochemistry revealed increased mPGES immunoreactivity in endometriosis samples compared with eutopic endometria. Microsomal PGES immunoreactivity was observed in both epithelial cells and stromal or inflammatory cells of endometriosis. Increased expression of mPGES-1 mRNA was detected in most of the endometriosis samples.

Conclusion
Our results suggest that expression of mPGES in addition to COX-2 plays a role in increasing PGE_2 production in endometriosis.

Introduction

Endometriosis is a multifactorial complex disease process characterized by the ectopic presence of endometrial glands and stroma. It can present as peritoneal disease, endometriotic ovarian cysts, and/or deeply infiltrating rectovaginal endometriosis and is associated with pelvic pain, adhesion formation, and infertility. Endometriosis occurs in 30–40% of women with infertility and progressive disease in 40–50% of reproductive-aged women.[1,2] Ectopic endometrium has been reported to produce prostaglandins (PG) and may contribute to the pathophysiology of endometriosis.[3,4] Van Voolhis

et al.[5] used monoclonal antibody produced against sheep prostaglandin H (PGH) synthase and reported that a higher percentage of glands in endometriosis and adenomyosis contained PGH synthase, suggesting that these tissues are sites of PG synthesis. Downstream of the cyclooxygenase (COX) enzyme isoforms, the PGH_2 product can be further metabolized into various physiologically important eicosanoids such as PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin (PGI_2), and thromboxan A_2 by a number of specific synthases. Patients with endometriosis or adenomyosis have shown elevated levels of PG in the peritoneal fluid compared with controls.[4,6] Ota et al. determined the distribution of COX-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis,[7] and we previously reported the presence of upregulated messenger RNA (mRNA) levels in endometriosis lesions,[8] suggesting that enhanced synthesis of PGs as a consequence of upregulated COX-2 may be involved in the pathogenesis of endometriosis and the disease progression. The overexpression of COX-2 and PGE_2 synthesis in rat intestinal epithelial cells is reported to increase their proliferation rate, resistance to apoptosis, and invasiveness by suppressing the transcription of target genes that may be involved in cellular growth/transformation and adhesion.[9] Several arachidonic acid metabolites or eicosanoids are potent immunoregulatory molecules, which have been shown to modulate lymphocyte activation, cytokine production, and cytotoxic T lymphocyte (CTL) responses.

Recently, an inducible microsomal human PGE synthase (mPGES) was identified as another rate-limiting enzyme in PG synthesis.[10] This enzyme converts the COX product, PGH_2, to PGE_2, an eicosanoid that has been linked to carcinogenesis. Increased amounts of PGE_2 have been observed in many tumor types including colorectal adenomas and cancers, suggesting its possible involvement in gastrointestinal carcinogenesis.[11] The expression of mPGES has been identified in intestinal type gastric adenocarcinoma and in gastric cancer cell lines,[12] and mPGES expression has been identified in epithelial cells of endometrial adenocarcinoma.[13] COX-2 and mPGES have been reported to be functionally linked,[14] which suggests that aberrant mPGES expression could lead to elevated levels of PGE_2 in endometriosis. We investigated the expression of mPGES to elucidate the mechanism responsible for the observed elevation in the levels of PGE_2 in endometriosis and in its malignant transformation.

Materials and methods

Patient Samples

Tissue sections were obtained from women undergoing laparotomy or laparoscopic surgery in Nihon University Itabashi Hospital between 2004 and 2006. Ovarian specimens ($n = 28$; age range, 22–41 years; mean age ± SD, 29.54 ± 5.76) were obtained from women with ovarian endometriosis. Endometrial samples ($n = 12$; age range, 30–44 years; mean ± SD, 36.38 ± 4.73) obtained by hysterectomy for other diseases, including uterine myoma and localized cervical malignancies, were designated as controls. In each case, the menstrual cycle day was determined by histologic dating.

Tissue sections were cut from the ovarian cyst wall including the epithelium, and the uterine body, and were then formalin fixed and paraffin embedded for immunohistochemical analysis. Thirteen specimens were prepared for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mPGES-1 and COX-2, and were stored at −80°C. Informed consent was obtained from all patients. Clinical data were obtained by a retrospective review of the medical records.

Immunohistochemistry

We used rabbit mPGES-1 anti-human affinity-purified polyclonal antibody purchased from Cayman Chemical (Ann Arbor, MI, USA), and sections were stained using a Histofine Simple Stain MAX PO(R) kit (Nichirei Biosciences, Inc., Tokyo, Japan). Surgical specimens were fixed with neutral-buffered 10% formalin, processed, and the embedded in paraffin. Sections were cut (thickness, 4 μm) and mounted on aminopropyl triethoxysilane (APS)-coated slides. The dried slides were deparaffinized in xylene and rehydrated through an ethanol series. The slides were then immersed in 0.3% hydrogen peroxidase in methanol for 30 min, to deplete endogenous peroxidase, and exposed to microwave radiation, to activate their antigenicity. Sections were cooled for 5 min, and incubated with protein-blocking solution for 10 min. Staining was performed using antiserum to mPGES-1 at a 1:500 dilution for 60 min at 37°C. Bound antibodies were detected with the Simple Stain MAX PO(R) reagent, using diamino-benzidine tetrahydrochloride as the substrate, and the sections were counterstained with hematoxylin. Negative

controls were prepared by omission of the primary antibody.

In addition, to visualize positive cells in stroma clearly, immunohistochemistry was also carried out the same procedure with rabbit mPGES-1 anti-human polyclonal antibody and goat antirabbit IgG conjugated with peroxidase-labeled dextran as the secondary antibody. The section was visualized using 3-amino-9-ethyl carbazole.

Slides were independently interpreted by two observers. Staining for mPGES-1 was graded as 3 for strongly reactive staining, 1 for weakly reactive but with specific staining, 2 for staining that was between 1 and 3, and 0 for no reactivity. Statistical analysis was performed by the Mann–Whitney U test. $P < 0.05$ was considered to be statistically significant.

RNA Isolation and RT

Tissue samples were snap-frozen with a spray freezer, and the RNA was extracted after homogenization. RNA was extracted by the acid guanidinium–

Table I Oligonucleotide Primers Used for the Reverse Transcriptase Polymerase Chain Reaction

Target gene	Product length (bp)	Primer (5′–3′)	Annealing temperature (°C)	Cycle
mPGES-1[a]	502	GCAGCTGACCCTGTACCAG TTGACTACATTGTCCGCGAG	57	30
COX-2[b]	243	TTCAAATGAGATTGTGGGAAAAT AGATCATCTCTGCCTGAGTATCTT	60	35
GAPDH[c]	571	ATTCCATGGCACCGTCAAGGCT TCAGGTCCACCACTGACACGTT	58	30

mPGES-1, microsomal human prostaglandin E synthase-1; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[a]Yuko Kino et al.[15].
[b]Hla and Neilson[16].
[c]Adcock et al.[17].

Table II mPGES-1 Immunohistochemical Staining of Eutopic Endometrium (UEM)

Case no.	Age	Diagnosis	Menstrual cycle (cycle day)	mPGES staining: Epithelial glands	mPGES staining: Stromal cells
UEM-1	32	CCIb	Early secretory (17)	0	1
UEM-2	37	CIS	Secretory (25)	0	1
UEM-3	40	CCIb	Secretory (19)	0	1
UEM-4	41	CCIa	Secretory (22)	0	0
UEM-5	34	CCIb	Secretory (23)	0	1
UEM-6	30	CCIb	Secretory (26)	0	1
UEM-7	32	CCIb	Secretory (25)	0	1
UEM-8	34	CCIb	Secretory (27)	0	1
UEM-9	43	CIS	Secretory (23)	0	0
UEM-10	33	CIS	Proliferative (10)	0	1
UEM-11	44	Myoma	Proliferative (8)	0	1
UEM-12	39	Myoma	Proliferative (13)	0	0
UEM-13	37	CCIb	Proliferative (12)	0	2

Staining was graded as 3 for strongly reactive staining, 1 for weakly reactive, but still specific staining, 2 for staining between 1 and 3, and 0 for no reactivity.
mPGES-1, microsomal human prostaglandin E synthase-1; CIS, carcinoma *in situ*; CC, cervical carcinoma; myoma, uterine myoma.

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phenol–chloroform method using the RNA zol™ (Leedo Laboratories, Inc., Houston, TX, USA) rapid RNA purification kit. Total RNA solutions were adjusted to a concentration of 1 mg/mL with diethyl pyrocarbonate-treated water and stored at −70°C until used. A single-strand c-DNA copy was made from 1 μg of total RNA using a random hexamer and MMTV RTase (First Strand cDNA synthesis kit; Pharmacia Tokyo, Tokyo, Japan).

Polymerase Chain Reaction

To minimize any non-specific reaction, we employed the 'hot start' technique for the PCR. Twenty-five microliters of the following reaction mixture was used for the PCR: 13 μL of HotStar Taq Master Mix (this solution provides a final concentration of 1.5 mM $MgCl_2$ and 200 μM of each deoxynucleotide triphosphate; Qiagen, Tokyo, Japan), 1 μL of each primer (20 pmol each), 10 μL of distilled water, and 1 μL of the reverse-transcribed cDNA sample. The mixture was heated for 3 min at 94°C, and again for 3 min at 60°C. It was then subjected to 30 or 35 cycles of 60 s at 94°C; 60 s at either 57, or 58, or 60°C; and 60 s at 72°C using a thermal cycler (Perkin-Elmer, Cetus Tokyo, Japan). Table I describes the primers[15–17] used to detect mPGES and COX-2 mRNA. Ten microliters of PCR products were then separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Table III Microsomal Human Prostaglandin E Synthase-1 (mPGES-1) Immunohistochemical Staining of Ovarian Endometriosis (OVEM)

			mPGES staining	
Case no.	Age (years)	Reproductive history	Epithelial glands	Stromal cells
OVEM-1	27	0g0p	3	1
OVEM-2	30	3g1p	2	1
OVEM-3	35	2g1p	1	2
OVEM-4	26	0g0p	2	2
OVEM-5	28	0g0p	3	2
OVEM-6	27	0g0p	3	2
OVEM-7	35	0g0p	2	1
OVEM-8	28	0g0p	3	1
OVEM-9	37	2g1p	1	0
OVEM-10	37	2g1p	1	0
OVEM-11	26	0g0p	2	2
OVEM-12	31	0g0p	1	1
OVEM-13	37	1g1p	3	2
OVEM-14	22	0g0p	2	1
OVEM-15	25	0g0p	2	2
OVEM-16	30	0g0p	2	1
OVEM-17	31	0g0p	3	2
OVEM-18	41	0g0p	1	1
OVEM-19	22	0g0p	2	1
OVEM-20	31	0g0p	1	1
OVEM-21	38	4g3p	0	1
OVEM-22	23	0g0p	0	1
OVEM-23	35	0g0p	1	1

Staining was graded as 3 for strongly reactive staining, 1 for weakly reactive but still specific staining, 2 for staining between 1 and 3, and 0 for no reactivity.

Results

Tables II and III present the results of immunohistochemical staining of the eutopic endometrial and ovarian endometriotic tissues. mPGES-1 was not expressed in epithelial cells of eutopic endometria, in both the proliferative and secretory phases (Table II). Some endometrial stromal cells (arrow) were stained, although the majority was negative (Fig. 1). These positive stromal cells were considered to be of fibrohistiocytic cells, based on their morphologic features.

We also observed increased immunoreactivity of mPGES in endometriosis samples compared with eutopic endometria (Table III). In 21 of 23 cases, mPGES-1 was expressed in the lining epithelial cells. Immunoreactivity score of mPGES-1 in epithelial glands of endometriosis was significantly pronounced than that of eutopic endometrium ($P < 0.0001$). On the other hand, we observed increased immunoreactivity score of mPGES-1 in stromal cells of endometriosis compared with that of eutopic endometrium, but there were no significant difference ($P = 0.051$). In a case of ovarian endometriosis, mPGES-1 was strongly expressed in the lining epithelial cells (Fig. 2a,b). Furthermore, immunoreactivity of mPGES-1 was most remarkable in the epithelia of an endometrial cyst with borderline malignancy [ovarian endometriosis (OVEM-1)] (Fig. 3).

The expression of mPGES-1 mRNA was either weak or not present in eutopic endometria compared with ovarian endometriosis (Fig. 4). Intense mPGES-1 mRNA expression was observed in a case of endometrial cyst with border-line malignancy (Fig. 5, Lane 2).

Table IV shows the results of mPGES-1 and COX-2 mRNA expression in ovarian endometriosis cases. A coincidental expression of COX-2 and mPGES-1

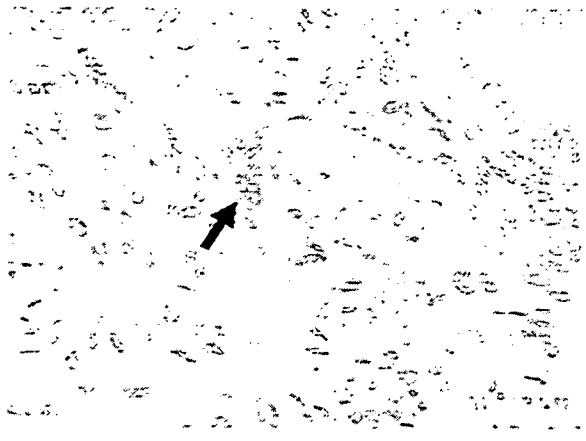

Fig. 1 Microsomal human prostaglandin E synthase-1 was not expressed in epithelial cells of eutopic endometria. Some endometrial stromal cells (arrow) were stained, although the majority was negative (secretory phase, immunostaining, original magnification: ×400). Color development was performed with 3-amino-9-ethyl carbazole. These positive stromal cells were considered to be of fibrohistiocytic cells, based on their morphologic features.

mRNA was observed in most cases of ovarian endometriosis. Interestingly, we noted a discrepancy in the extent of upregulation of mPGES and COX-2. OVEM-21 and OVEM-26, for example, were positive for the expression of COX-2 but not for mPGES; and this pattern was reversed in OVEM-23.

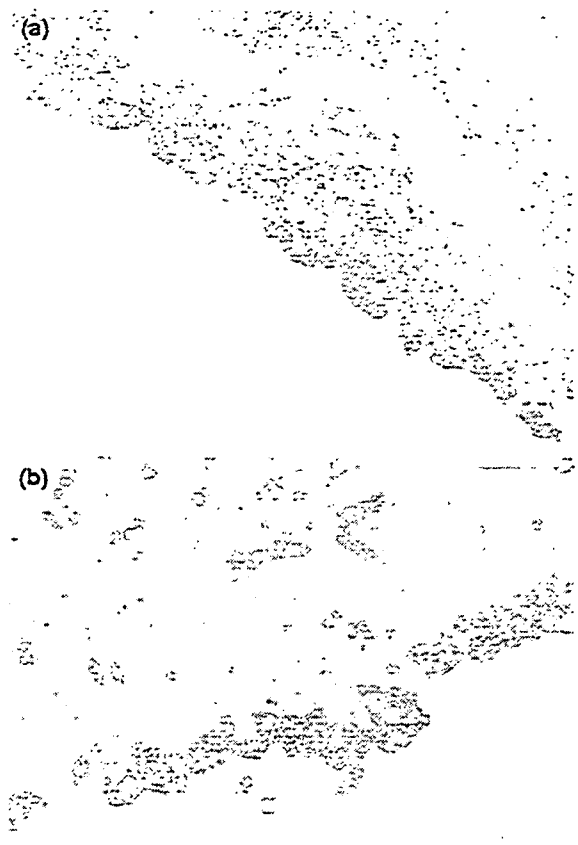

Fig. 2 Microsomal prostaglandin E synthase-1 is strongly expressed in epithelial cells in a case of ovarian endometriosis [immunostaining, original magnification: (a) ×100; (b) ×400].

Discussion

In the present study, microsomal PGES immunoreactivity was observed in both epithelial cells and stromal or inflammatory cells of endometriosis. mPGES-1 was not expressed in epithelial cells of eutopic endometria. This result indicates that at least some of the excess PGE is originated from epithelial glands of endometriosis in addition to stromal cells. We found that mPGES mRNA was expressed in cases of endometriosis, and that mPGES immunoreactivity in endometriosis increased in comparison with that in eutopic endometria. As COX-2 is known to be overexpressed in the majority of endometriosis cases,[7,8] it is likely that enhanced expression of mPGES in addition to COX-2 contributes to the increased levels of PGE_2 in peritoneal fluid in patients with endometriosis or adenomyosis[4,6] that may be associated with pelvic pain, adhesion formation, and infertility.

Marked differences in the extent of upregulation of mPGES and COX-2 were observed in some endometriosis cases. This suggested that the mechanisms controlling the expression of these two enzymes are different, as seen in colorectal cancers.[11] On the basis of the known sequence of the mPGES promoter,[18] it is uncertain whether either of these elements plays a significant role in regulating mPGES transcription. While the mPGES promoter lacks a TATA box, the COX-2 promoter does not.[19] Another explanation is that the amount of supplied arachidonic acid may be different in endometriosis cases. Because increased supply of arachidonic acid by explosive activation of cytosolic phospholipase A_2 mPGES to be coupled with COX-1, rather than with COX-2.[14]

Although generally acknowledged as a benign disease, endometriosis has many features in common with neoplasia, such as clonal proliferation.[20–23]

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Fig. 3 The immunoreactivity of microsomal prostaglandin E synthase-1 was more marked in the epithelia of a case (OVEM-1) of endometrial cyst with border-line malignancy (immunostaining, original magnification ×200).

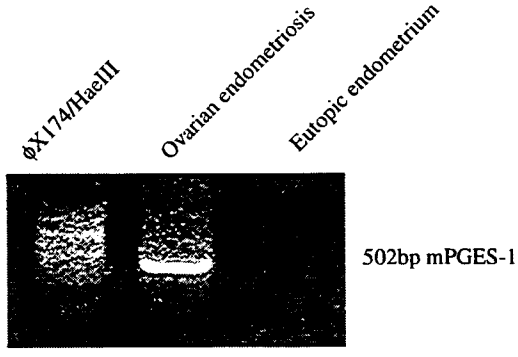

Fig. 4 The expression of microsomal prostaglandin E synthase-1 messenger RNA was weak or absent in eutopic endometrium compared with ovarian endometriosis.

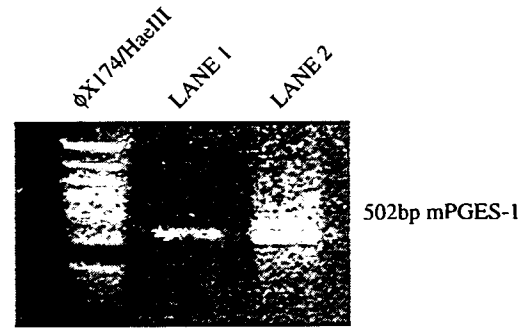

Fig. 5 Microsomal prostaglandin E synthase (mPGES-1) messenger RNA (mRNA) expression was observed in representative case (OVEM-13) of endometrial cyst (lane 1). Intense mPGES-1 mRNA expression was observed in a case (OVEM-1) of endometrial cyst with border-line malignancy (lane 2).

Table IV Expression of Microsomal Human Prostaglandin E Synthase-1 (mPGES-1) and Cyclooxygenase-2 (COX-2) Messenger RNA by Reverse Transcriptase Polymerase Chain (RT-PCR) Reaction in Ovarian Endometriosis

Case no.	Age	Reproductive history	COX-2 mRNA	mPGES-1
OVEM-1	27	0g0p	+	+
OVEM-13	37	1g1p	+	+
OVEM-14	22	0g0p	−	−
OVEM-18	41	0g0p	−	−
OVEM-20	31	0g0p	−	−
OVEM-21	38	4g3p	+	−
OVEM-23	35	0g0p	−	±
OVEM-24	22	0g0p	+	+
OVEM-25	34	1g1p	+	+
OVEM-26	25	0g0p	+	−
OVEM-27	22	0g0p	+	+
OVEM-28	22	0g0p	+	+

Five cases of OVEM-24, OVEM-25, OVEM-26, OVEM-27, and OVEM-28 were prepared for RT-PCR analysis only.

Women with a history of endometriosis are at an increased risk for developing ovarian cancer, particularly endometrioid and clear cell carcinoma.[24] Furthermore, 15–40% of endometrioid ovarian carcinoma cases are associated with endometriosis.[25] The possibility that ovarian cancer arises through the malignant transformation of endometriosis has long been suggested. Denkert et al.[26] reported that COX-2 expression was increased in ovarian cancer cells and that it was an important prognostic factor. In the present study, we found markedly more immunoreactivity of mPGES-1 in the epithelia, but not in the papillary growing epithelia, of an endometrial cyst in a case with border-line malignancy. mPGES-1 may play a critical role in tumor development and in inducing malignant transformation in endometrial cysts instead of in cancer cell proliferation.

As elevated levels of COX-2 and PGE_2 have also been observed in ulcerative colitis and Crohn's disease, both chronic inflammatory diseases that predispose to colon cancer,[27,28] it is important to determine whether mPGES is overexpressed in such inflammatory conditions. Ovarian endometriosis is another example of a chronic inflammatory disease. Non-physiologic sources of inflammation include an