Monoamine oxidase A is highly expressed by the human corpus luteum of pregnancy

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Abstract

To investigate the physiological characteristics of the corpus luteum (CL) of pregnancy, we raised a mAb, human corpus luteum (HCL)-4, against human luteal cells obtained from CL of pregnancy. The affinity-purified antigen from human CL of pregnancy or placenta using HCL-4 was a 61 kDa protein. The partial amino acid sequence of the antigenic protein was identical to that of human monoamine oxidase A (MAOA, EC1.4.3.4). MAOA has been shown to catabolize catecholamines that were reported to regulate luteal function in CL and vasoconstriction in various organs. Immunohistochemistry using HCL-4 mAb showed that MAOA was intensely expressed on large luteal cells and moderately expressed on small luteal cells in the CL of pregnancy. In the CL of menstrual cycle, MAOA was weakly detected on large luteal cells but not detected at all on small luteal cells. Western blotting analysis confirmed the high expression of MAOA in CL of pregnancy. Northern blot analysis also showed the expression of MAOA mRNA in human CL, and showed that its expression was higher in CL of pregnancy than in CL of menstrual cycle. The increased expression of MAOA in the CL of pregnancy suggests the contribution of MAOA to the function of the CL of pregnancy.

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Introduction

During normal pregnancy, the corpus luteum (CL) of pregnancy is formed from CL of menstrual cycle when the embryo implants in the uterus. In the human CL of pregnancy, luteal cells become hypertrophic and produce abundant steroid hormone until at least 7–9 weeks of gestation to maintain embryo implantation (Adams & Hertig 1969, Csapo *et al.* 1973, Yen 1991). Thus, the CL of pregnancy is an essential organ in early pregnancy, and its biochemical and/or functional analyses are important to understand the precise mechanisms of human reproduction.

We previously reported that the expression profiles of several cell surface molecules on luteal cells became changed from CL of menstrual cycle to CL of pregnancy. For example, membrane-bound aminopeptidase-N and dipeptidyl peptidase-IV were highly expressed on small luteal cells in the CL of menstrual cycle, but it was hardly detected on small luteal cells in the CL of pregnancy (Fujiwara *et al.* 1992a, 1992b). Other membrane-bound peptidases, carboxypeptidase-M and endothelin-converting enzyme-1, expressed their isotypes only in CL of pregnancy (Yoshioka *et al.* 1998a, 1998b), suggesting that these peptidases are involved in the function of CL of pregnancy (Fujiwara 2004). On the other hand, integrin $\alpha 6$ was expressed on large luteal cells in the CL of menstrual cycle, but it was not expressed on those in the CL of pregnancy

(Honda *et al.* 1995). In addition to these cell surface molecules, relaxin gene expression was reported to increase in the CL of pregnancy (Bogic *et al.* 1995). Furthermore, we observed that the responses to immune cells in the luteal cell cultures were different between women in mid-luteal phase and early pregnancy. In the luteal cell culture derived from CL of pregnancy, interleukin 4 (IL4) and IL10 productions by the immune cell stimulation were significantly higher than those in the culture derived from CL collected during the mid-luteal phase. In addition, IL4 and IL10 promoted progesterone production by luteal cells as much as human chorionic gonadotropin (HCG) *in vitro* (Hashii *et al.* 1998). These results supported that human CL of pregnancy is functionally different from CL in mid-luteal phase and proposed that the CL function is regulated not only by HCG (Takao *et al.* 1997) but also by immune cells (Fujiwara 2006).

From these findings, it is speculated that CL of pregnancy is in a further differentiated stage compared as CL of menstrual cycle. However, there has been no definite evidence to conform this concept. Therefore, in this study, to identify the key substances that are specifically involved in the physiology of CL of pregnancy, we raised mAbs against human CL of pregnancy and obtained a mAb, named human corpus luteum (HCL)-4, which highly reacted with cytoplasmic molecules in luteal cells in the CL of pregnancy.

The subsequent analysis of purified antigen demonstrated that HCL-4 mAb detects monoamine oxidase A (MAOA, EC1.4.3.4). Since MAOA catabolizes biogenic amines such as dopamine, norepinephrine, and epinephrine that are well known to regulate vasoconstriction, this enzyme can be one of the crucial molecules to regulate the function of CL of pregnancy. Therefore, we further examined the precise expression profiles of MAOA in human follicles, CL of menstrual cycle, and CL of pregnancy in relation to their differentiation stages.

Results

The expression profiles of HCL-4 antigen in human follicles and CL

One hybridoma was selected by immunohistochemistry. This mAb was named HCL-4 and belonged to the IgG3 isotype.

In growing follicles, 4–5 mm in diameter, HCL-4 antigen was rarely detected on granulosa or theca interna cells (Fig. 1A–C). In two of three preovulatory follicles, 18–20 mm in diameter, HCL-4 antigen was weakly detected on theca interna cells but not on the granulosa cells. In the remaining one preovulatory follicle, HCL-4 antigen was not detected on the granulosa cells or theca interna cells (data not shown).

In CL of the early luteal phase, HCL-4 antigen was weakly detected in the luteinizing granulosa/large luteal cells in three out of seven CL. In the residual four CL, HCL-4 antigen was not expressed on luteinizing granulosa/large luteal cells. In CL of the mid-luteal phase, large luteal cells weakly expressed HCL-4 antigen in three out of six CL (Fig. 1D–F), but not in the residual 3 CL. In CL of the late luteal phase, large luteal cells weakly expressed HCL-4 antigen in two out of three CL examined, but not in the third CL. In all menstrual CL, there was no HCL-4 antigen detected on luteinizing theca interna/small luteal cells.

In CL of pregnancy at 6–15 weeks of gestation, HCL-4 antigen was highly expressed on large luteal cells and weakly or moderately expressed on small luteal cells (Fig. 1G–I). It was not expressed in ovarian stromal cells or blood vessels.

The intensity score of HCL-4 antigen expression on large luteal cells in CL of pregnancy was significantly higher than that of CL of menstrual cycle ($P < 0.01$). HCL-4 antigen expression on small luteal cells in CL of pregnancy was also significantly higher than that of CL of menstrual cycle ($P < 0.01$; Table 1).

Purification of HCL-4 antigen from a CL of pregnancy

The antigenic molecule was affinity purified from a CL of the menstrual cycle (CL day 9) and a CL of pregnancy at 10 weeks of gestation. SDS-PAGE profile showed that

Figure 1 HCL-4 antigen expression detected by indirect immunofluorescence staining. (A–C) A growing follicle (5 mm in diameter); (D–F) a CL on day 8; (G–I) a CL at 10 weeks of gestation. (A, D and G) Hematoxylin and eosin staining; (B, E and H) staining with HCL-4 mAb; (C, F and I) negative control stained with anti-TNP mAb. (A–C) HCL-4 antigen was not detected on granulosa cells (GC) or theca interna cells (TI). (D–F) HCL-4 antigen was weakly expressed on large luteal cells (LL) and not expressed on small luteal cells (SL). (G–I) HCL-4 antigen was intensely expressed on the cytoplasmic region of large luteal cells and moderately expressed on small luteal cells. Dotted lines show small luteal cell area. V, vessels; Str, stroma cells. Bar shows 100 μ m.

Table 1 HCL-4 antigen expression in human follicles and corpus luteum (CL) detected by indirect immunohistochemistry.

	GC/LL	TI/SL
Growing follicles (4–5 mm, <i>n</i> =3)	–	–
Preovulatory follicles (18–20 mm, <i>n</i> =3)	–	±
CL of menstrual cycle		
Early luteal phase (<i>n</i> =7)	± ^a	– ^c
Mid-luteal phase (<i>n</i> =6)	± ^a	– ^c
Late luteal phase (<i>n</i> =3)	± ^a	– ^c
CL of pregnancy (<i>n</i> =11)	+++ ^b	++/+ ^d

GC, granulosa cells; TI, theca interna cells; LL, large luteal cells; SL, small luteal cells; + + +, intensely positive (score 3); + +, moderately positive (score 2); +, weakly positive (score 1); –, negative (score 0); ±, some samples were weakly positive and the others were negative. The expression intensity of HCL-4 antigen on LL in CL of pregnancy was significantly higher than that of CL of menstrual cycle (b versus a; $P < 0.01$). HCL-4 antigen expression on SL in CL of pregnancy was also significantly higher than that of CL of menstrual cycle (d versus c; $P < 0.01$).

HCL-4 antigen was a single protein band of molecular mass of 61 kDa (Fig. 2). The staining of 61 kDa protein from CL of pregnancy was much higher than that from CL of menstrual cycle.

Figure 2 Silver-stained 10% SDS-PAGE profile of HCL-4 antigen purified from a CL on day 9 and a CL at 10 weeks of gestation. Lane 1, purified proteins from a CL on day 9 using HCL-4 mAb by immunoaffinity chromatography. Lane 2, purified proteins from a CL at 10 weeks of gestation using HCL-4 mAb by immunoaffinity chromatography. Lane 3, purified proteins from a CL on day 9 using anti-TNP mAb (negative control). Lane 4, purified proteins from a CL at 10 weeks of gestation using anti-TNP mAb (negative control). A specific protein band was observed at 61 kDa (arrow head). The amount of the 61 kDa protein from CL of pregnancy was much higher than that from CL of menstrual cycle. Bars show molecular mass markers of 200, 116, 97, 66.2, 45, and 31 kDa from the top to bottom.

The expression profiles of HCL-4 antigen in various human tissues

HCL-4 antigen was highly expressed in syncytiotrophoblasts of human chorion and placenta (Fig. 3A). It was also expressed in hepatocytes of the human liver. The antigen was hardly detected in mononuclear cells in peripheral blood (data not shown).

Purification of HCL-4 antigen from placenta and partial amino acid sequencing of the protein

The antigenic molecule was affinity purified from placenta. SDS-PAGE profile showed that the antigenic molecule exhibited the same molecular mass of 61 kDa as that from CL (Fig. 3C). N-terminal amino acid sequencing of this antigenic molecule was not performed successfully. SDS-PAGE profile of the purified molecule treated with lysyl endopeptidase showed a main protein fragment in the range of molecular mass of 12 kDa. The sequence of 20 amino acids from the N-terminus of this protein fragment was identical to

Figure 3 HCL-4 expression in term placenta and silver-stained 12% SDS-PAGE profile of HCL-4 antigen purified from term placenta. (A and B) Sequential frozen sections of placenta at 38 weeks of gestation derived from normal delivery. (A) HCL-4 was expressed on syncytiotrophoblast of chorionic villi (CV). (B) Negative control stained by anti-TNP mAb. Bar shows 100 µm. (C) Silver staining of HCL-4 antigen purified from term placenta. Lane 1, purified proteins from a term placenta using HCL-4 mAb by immunoaffinity chromatography. Lane 2, purified proteins from a term placenta using anti-TNP mAb (negative control). A specific protein band was observed at 61 kDa (arrow head). Bars show molecular mass markers of 200, 116, 97, 66.2, 45, 31, 21.5, and 14.4 kDa from the top to bottom.

that from the 31st amino acid to the 50th amino acid of human MAOA. It also showed 70% homology with the sequence from the 22nd to the 41st amino acid of MAOB (Fig. 4).

Western blotting analysis of MAOA in human CL and term placenta

Proteins from CL of the mid-luteal phase (CL on day 8) or of pregnancy (7 weeks of gestation) were electrophoresed, and MAOA was visualized with rabbit-specific antibody. Specific protein bands were detected in both CL of menstrual cycle and pregnancy, which accorded with MAOA protein bands in the placenta (Fig. 5A). The MAOA expression was significantly higher in CL of pregnancy (7, 7, and 12 weeks of gestation), compared with that of mid-luteal phases (CL on days 7, 8, and 9, $P < 0.01$; Fig. 5B and C).

MAOA and von Willebrand factor expressions in a CL of pregnancy

HCL-1 antigen was specifically expressed on large luteal cells, while it was not detected on 3 β -hydroxysteroid dehydrogenase (HSD3B)-positive small luteal cells (Fig. 6B). MAOA was expressed on both HSD3B-positive large and small luteal cells (Fig. 6C). On the other hand, there was no MAOA expression detected on large or small vessels, which were positively stained by anti-human von Willebrand factor polyclonal antibody (pAb; Fig. 6D).

The expression of MAOA mRNA in human CL

Both the 4.4 kbp main bands and 2.1 kbp weak bands of MAOA mRNA were detected by northern blotting of mRNA respectively isolated from corpus lutea of the mid-luteal phase and pregnancy (Fig. 7A). The MAOA mRNA expression was significantly increased in CL of pregnancy, compared with that of mid-luteal phases ($P < 0.05$), in accord with the immunohistochemical results described above (Fig. 7B).

HCL-4 antigen fragment: LLTEYGVSVLVLEARDRVGG

	11	1	21	31
MAOA:	MENQEASIA	GHMFDVVIG	GGISGLSAK	LLTEYGVSVL VLEARDRVGG
MAOB:	MSNKCDVVVV	GGGISGMAAA	KLHDSGLNV	VVLEARDRVG GRITYTLRNQK

Figure 4 Partial amino acid sequences of the 12 kDa protein fragment derived from HCL-4 antigen, MAOA and MAOB. The sequence of 20 amino acids from N-terminus of 12 kDa protein fragment was identical to that from the 31st amino acid to the 50th amino acid of human MAOA and showed 70% homology with the sequence from the 22nd to the 41st amino acid of MAOB (Bach *et al.* 1988). *Amino acid residues that were identical with the HCL-4 antigen fragment.

Figure 5 Western blotting analysis of MAOA in human corpora lutea. (A) Proteins from CL of mid-luteal phase (CL on day 8; lane 1) or of pregnancy (CL of 7 weeks of gestation; lane 2), and from chorion of term placenta (lane 3) were electrophoresed and blotted on PVDF membrane. MAOA was stained with rabbit-specific antibody. Arrowhead represents MAOA, and bars show gel top and molecular mass markers of 250, 150, 100, 75, 50, and 37 kDa from the top to bottom. The staining of MAOA was clearly observed in CL of day 8 and early pregnancy (lanes 1 and 2). (B) Proteins from CL of mid-luteal phase (CL on days 7, 8, and 9; lanes 1–3) or of pregnancy (CL of 7, 7, and 12 weeks of gestation; lanes 4–6) were electrophoresed and stained with anti-MAO (arrowheads) and anti- β -actin (arrows) pAbs. (C) Relative intensity (MAOA/ β -actin) in Fig. 5B was significantly higher in proteins derived from CL of pregnancy than those from CL of mid-luteal phase ($P < 0.01$).

Discussion

MAO (EC1.4.3.4) is the membrane-bound enzyme that catabolizes a variety of biogenic amines and is widely distributed in the central nervous system (Berry *et al.* 1994). MAO includes two types of isozyme, MAOA metabolizing mainly serotonin or norepinephrine and MAOB metabolizing mainly tyramine. The calculated molecular weights of MAOA and -B are 59 700 and 58 800 respectively and both the enzymes share a 70% homology of amino acid sequences (Bach *et al.* 1988). MAOA was reported to be expressed mainly in catecholaminergic neurons in the brain and other tissues including the placenta, liver, and skin. MAOB, on the other hand, was reportedly expressed mainly in serotonergic and histaminergic neurons in the brain, liver, skin, lymphocytes, and placenta (Berry *et al.* 1994, Auda *et al.* 1998, Shih *et al.* 1999, Rodriguez *et al.* 2001). Recently, it has been suggested that MAOs may play a role in inducing cancers (Agostinelli *et al.* 2004, Toninello *et al.* 2006).

In the present study, we raised mAb that highly reacted with cytoplasmic molecules in the luteal cells in CL of pregnancy. This mAb also reacted with syncytiotrophoblasts of placenta. The purified molecule from CL and placenta showed a molecular mass of 61 kDa, which corresponded to the calculated value for MAO (Bach *et al.* 1988) and accorded with that of MAOA (61 kDa) in adipose tissue (Pizzinat *et al.* 1999). The partial amino

Figure 6 HCL-4 antigen and von Willebrand factor expressions in a CL of pregnancy (7 weeks of gestation) detected by double staining. Sequential frozen sections were stained by (A) H-E attaining, (B) red-stained by rhodamine using HCL-1 and (C and D) HCL-4 mAbs, (B and C) and green-stained by FITC using anti-HSD3B, and (D) anti-von Willebrand factor pAbs. (B) HCL-1 antigen was specifically expressed on large luteal cells (LL), while it was not detected on HSD3B-positive small luteal cells (SL). (C) MAOA was expressed on both HSD3B-positive large and small luteal cells. (D) There was no MAOA expression detected on large or small vessels, which were positively stained with anti-human von Willebrand factor pAb. Bar shows 100 μ m. Dotted lines show small luteal cell area.

acid sequence of purified antigenic molecule from human placenta was completely identical to the sequence from the 31st to the 50th amino acid of MAOA. Although it also showed 70% homology with the sequence of MAOB, from the fact that HCL-4 mAb was not reactive to peripheral blood mononuclear cells or ovarian blood vessels that were reported to strongly express MAOB (Yoshimoto *et al.* 1986, Berry *et al.* 1994), it has been concluded that the HCL-4 mAb is specifically reactive to human MAOA.

Immunohistochemical study showed that immuno-reactive MAOA protein was abundantly expressed in the CL of pregnancy. The expression of MAOA on the large luteal cells in CL of menstrual cycle remained constantly low throughout the luteal phase. MAOA expression on the small luteal cells during menstrual cycle was not detected although weak expression was observed in the theca interna cells in the preovulatory follicles. The apparent difference in MAOA protein expression between CL of menstrual cycle and pregnancy was also shown by immunoaffinity chromatography (Fig. 2). In addition, western and northern blot analyses showed high expression of MAOA protein at 61 kDa and mRNA at 4.4 and 2.1 kbp in CL of pregnancy (Figs 5 and 7). These profiles of MAOA mRNA accord with the reported ones that were derived from the adipose tissue (Pizzinat *et al.* 1999). Taken together with the profiles obtained by immunohistochemistry and SDS-PAGE, we conclude

Figure 7 Northern blotting analysis of MAOA mRNA in human corpora lutea. (A) mRNAs from menstrual corpora lutea (on CL days 7, 8, 8, and 9; lanes 1–4) and pregnant corpora lutea (at 7, 7, 9, and 12 weeks of gestation; lanes 5–8) were subjected to northern blotting analysis using probes for MAOA (upper panel) and ribosomal protein S26 (lower panel). The 4.4 kbp main bands and 2.1 kbp weak bands of MAOA mRNA were detected in the mRNAs isolated from both CL of mid-luteal phases and pregnancy. (B) The level of MAOA mRNA was determined by densitometric scanning of the autoradiograph from the northern blot analysis for 8 CL (lane 1, CL of mid-luteal phase, $n=4$; lane 2, CL of pregnancy, $n=4$). The MAOA mRNA level was corrected with RPS26 mRNA expression. The MAOA mRNA expression of CL of pregnancy was significantly higher than that of CL of mid-luteal phase ($P<0.05$).

that MAOA expression increases in CL of pregnancy, supporting the concept that CL of pregnancy is in the further differentiated state from CL of menstrual cycle. However, it should be noted that because these CL samples were obtained from patients having uterine myoma and benign ovarian cysts, these diseases may anatomically influence vascular circulation and ovarian function affecting MAOA expressions. In northern blot analysis, MAOA mRNA expression in the CL derived from 12 weeks of gestation, when luteo-placental shift has already occurred (Schindler 2005), was relatively high. Although the precise reasons are unclear, there is discrepancy between the expression intensity among western (Fig. 5) and northern blot analyses. This may be caused by unknown regulatory mechanisms for inherent expression of MAOA.

In the rat ovary, MAOA-like enzyme activity was detected in the CL of estrous cycle (Yoshimoto *et al.* 1986). The total MAO activity in the rat ovary was reported to gradually increase during pregnancy until delivery (Kono *et al.* 1994). It is widely known that MAO plays a protective role in various tissues by oxidizing amines in circulation. MAOA expressed in the syncytiotrophoblast layer in human placenta was suggested to protect against hypertension caused by catecholamines and serotonin in circulating blood, which arise in toxemia of pregnancy (Yoshimoto *et al.* 1986). Consequently, high expression of MAOA on the luteal cells of CL of pregnancy suggests that MAOA is involved in metabolizing biogenic amines within CL of pregnancy. By transvaginal color velocity imaging and pulsed Doppler ultrasonography, human CL blood flow was reported to remain constant during normal early pregnancy from 6 to 12 weeks of gestation (Salim *et al.* 1994, Alcazar *et al.* 1996). The vascular network in CL is established in the mid-luteal phase and further matured during early pregnancy. Innervation of peripheral nerve system, which contains a catecholamine system, may develop around newly constructed vessels. In perfused human ovaries, α -adrenergic agonists were shown to cause vasoconstriction, while β -adrenergic agonists cause vasodilation (Varga *et al.* 1979). Thus, MAOA expressed in the human CL of pregnancy is speculated to regulate luteal blood flow in early pregnancy by decreasing the biological activity of catecholamines.

Accumulating evidence has shown that catecholamines directly affect luteal cell function. Norepinephrine stimulation was reported to enhance progesterone secretion from bovine CL (Bogacki & Kotwica 1999). The presence of norepinephrine and dopamine was also demonstrated in bovine CL (Battista *et al.* 1989, Kotwica *et al.* 1996). Epinephrine was shown to promote progesterone and oxytocin concentration in goat CL as well (Cooke & Payne 1998). However, in the rabbit, catecholamines reduced the responsiveness of LH in the luteal cells (Jena & Abramowitz 1989). It was also reported that the administration of β -adrenergic receptor antagonist to pseudo-pregnant rabbit did not affect progesterone secretion (Gadsby *et al.* 1985). The direct effects of catecholamines on primate luteal cell function are controversial. It is assumed that the local concentrations and effects of catecholamines on CL must be taken into considerations. It was reported that epinephrine and norepinephrine stimulated progesterone production by cultured luteinizing human granulosa cells, which correspond to luteal cells in the early luteal phase (Webley *et al.* 1988). Although the precise mechanisms for direct effects of catecholamines on luteal cells are unknown, the high expression of MAOA in human CL of pregnancy suggests the physiological roles of catecholamines and MAOA in the function of CL of pregnancy.

In conclusion, this study indicated that MAOA was prominently expressed on large and small luteal cells in CL of pregnancy. The apparent increase in MAOA expression in CL from menstrual cycle to early pregnancy suggests that catecholamines play important roles in the function of CL of pregnancy.

Materials and Methods

Samples

Ovarian tissues were obtained from 30 women, aged between 28 and 43 years old. They had undergone unilateral ovarian cystectomy or oophorectomy and contralateral wedge resection to treat benign ovarian tumors. All women had a history of regular menstrual cycles (28–30 days) and their ovulatory basal body temperature charts showed normal luteal phase length. Macroscopically and microscopically, normal regions of these tissues were used for this study. The following follicles and CL were studied: three antral follicles that were growing follicles measuring 4–5 mm in diameter, three preovulatory follicles (18–20 mm in diameter), seven CL from CL day 2 (the day after ovulation) to day 5, six CL on days 6–10, and three CL on days 11–14. CL of pregnancy was obtained from 11 pregnant patients aged from 35 to 41 years old. Partial resection of CL of pregnancy was performed in ten pregnant women who underwent hysterectomy at 6 ($n=1$), 7 ($n=2$), 8 ($n=1$), 9 ($n=2$), 10 ($n=2$), 12 ($n=1$), and 14 ($n=1$) weeks of gestation due to uterine myoma ($n=8$) and/or uterine cervical carcinoma *in situ* ($n=2$, 7, and 9 weeks of gestation). One pregnant woman at 15 weeks of gestation underwent unilateral oophorectomy due to a huge mature cystic teratoma and a CL of pregnancy was obtained from the removed ovary. In all patients, fetal growth was normal on ultrasonographic examination. The CL day was re-evaluated according to the histological dating, using hematoxylin- and eosin-stained tissue sections from 10% (v/v) formalin-fixed and paraffin-embedded samples (Corner 1956). Human term placentae ($n=5$) were obtained from normal deliveries and human liver sample ($n=2$) was obtained from patients undergoing lobectomy for hepatoma. Peripheral blood mononuclear cells were also collected from volunteers as described previously (Kosaka *et al.* 2002). The use of these tissue samples was approved by the Ethics Committee of Kyoto University Hospital. The informed consent for use of these tissue samples was obtained from all the patients prior to the study according to the rule of Ethics Committee of Kyoto University Hospital.

mAb production

Luteal cells were isolated from CL tissue at 15 weeks of gestation as described previously (Fujiwara *et al.* 1996). Briefly, the CL was separated from the connective tissue, minced with scissors, and then incubated in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) containing 0.2% collagenase type I (Sigma Chemical Co.) and 0.01% DNase I (Sigma Chemical Co.) at 37 °C for 15 min. The cell suspension was incubated with 1000 IU/ml dispase (Godo Shusei Co. Ltd, Tokyo, Japan) in RPMI 1640 containing 10% fetal calf serum

(Flow Laboratories, McLean, VA, USA) at 37 °C for 30 min. Eight-week-old BALB/c mice were injected intraperitoneally with 3×10^6 human luteal cells three times with about 1-month interval. Under ethanol anesthesia, blood was taken from the immunized mice and then the spleen was removed after killing by cervical vertebrae dislocation. Spleen cells were isolated and fused with X63Ag8.653 myeloma cells using polyethylene glycol 1500 3 days after the last immunization (Köhler & Milstein 1975). Supernatants from the growing hybridomas were screened by indirect immunofluorescence staining of CL cryosections, which were prepared as described (Fujiwara *et al.* 1996). Briefly, fresh CL of menstrual cycle (CL on day 7) or CL of pregnancy (15 weeks of gestation) was embedded in OCT compound (Tissue-Tec; Miles Scientific, Naperville, IL, USA), snap-frozen in liquid nitrogen, and stored at -80 °C. Frozen tissues were cut into 7 μ m thick sections using a cryostatic microtome (Histostat, Reichert-Jung, Heidelberg, Germany). The sections were immediately and thoroughly air-dried on Neoplene (Nisshin EM Co. Ltd, Tokyo, Japan)-coated glass slides and then fixed with acetone at -20 °C. For screening, cryosections prepared from CL of pregnancy were incubated with supernatants of the growing hybridomas for 60 min at room temperature. After washing in PBS, specimens were incubated with FITC-conjugated rabbit anti-mouse pAb (diluted 1:50; DAKO Japan Co. Ltd, Kyoto, Japan) for 30 min at room temperature in the dark. The slides were washed, mounted with an anti-fade mounting agent (Perma Fluor Aqueous Mounting Medium; Immunon, Pittsburgh, PA, USA) to reduce fluorescence fading, and examined under a fluorescence microscope (Nikon, Tokyo, Japan).

The hybrid cells of interest were cloned twice by limiting the dilution method. The immunoglobulin isotype was determined using an isotyping kit for mouse mAbs (Serotec Ltd, Oxford, UK). The positive hybrid cells were injected intraperitoneally into mice previously treated with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, WI, USA). The IgG3 fraction was purified from ascitic fluid using Affi-Gel protein A (Bio-Rad Labs).

Immunohistochemical examination of HCL-4 antigen expression on CL and placenta

Purified HCL-4 mAb (5 μ g/ml, diluted in culture medium) was used for indirect immunofluorescence staining as described above. Anti-trinitrophenyl (TNP) mouse mAb (IgG3 class, 5 μ g/ml), which was an unrelated mAb raised against TNP, was used as the negative control (Tsujimura *et al.* 1990). The slides were washed, mounted with an anti-fade agent (Perma Fluor Aqueous Mounting Medium), and were examined within 6 h under a fluorescent microscope by two individuals. The intensity of antigen expression was graded from $-$ to $+++$ based on its fluorescence intensity and scored for statistical analysis ($-$, absence of staining, score 0; $+$, weak staining, score 1; $++$, medium staining, score 2; and $+++$, intense staining, score 3). If judgments differed, the lower value was selected. Two samples, a CL on day 8 (Fig. 1D–F) and a CL of pregnancy at 10 weeks of gestation (Fig. 1G–I), the fluorescence intensities of which were scores 1 and 3 respectively

were used as the standard visual assessment throughout this study as described previously (Fujiwara *et al.* 1994).

For double staining, the slides were incubated with HCL-1 mAb (5 μ g/ml), which specifically reacted with cell surface of large luteal cells in human CL (Fujiwara *et al.* 1996), HCL-4 mAb (5 μ g/ml), or negative control mAb (5 μ g/ml) for 60 min at room temperature. After washing in PBS, the slides were incubated with rhodamine-conjugated goat anti-mouse pAb (diluted 1:40; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 min at room temperature in the dark. After washing, FITC-conjugated sheep anti-human von Willebrand factor pAb (100 μ g/ml; Binding Site, Birmingham, UK) or FITC-conjugated sheep anti-rat IgG (negative control, 100 μ g/ml; Binding Site) was incubated for 30 min to detect the endothelial cells. After reacting with rhodamine-conjugated goat anti-mouse pAb, the other slides were incubated with rabbit anti-human HSD pAb (Oxygene, Dallas, TX, USA) or rabbit immunoglobulin (negative control) and then reacted with FITC-conjugated swine anti-rabbit pAb (DAKO, Glostrup, Denmark). After washing, the slides were mounted and examined under a confocal laser scanning microscope (Carl Zeiss, Inc., Jena, Germany).

Purification of the HCL-4 antigen from CL of pregnancy

Purification of the antigenic proteins was performed as previously reported (Honda *et al.* 1995). Tissue specimens (CL on day 9 or CL of pregnancy at 10 weeks of gestation, 0.5 g wet weight each) were homogenized in 5 ml of 40 mM phosphate buffer (pH 7.3) containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (Sigma Chemical Co.), 2 mM *p*-amidinophenylmethane sulfonylfluoride hydrochloride (Wako Pure Chemicals, Osaka, Japan), 10 μ g/ml leupeptin (Peptide Institute, Inc., Osaka, Japan), and 10 μ g/ml pepstatin (Peptide Institute, Inc.). After centrifugation (10 000 *g*, 30 min), the concentration of Nonidet P-40 was diluted to 0.3%. The supernatant was passed through a column containing 5 ml anti-TNP-conjugated Affi-gel 10 (Bio-Rad Labs; 2 mg IgG/ml gel) at 4 °C to remove nonspecifically bound components. The through-pass fraction was incubated with 0.1 ml HCL-4-conjugated Affi-gel 10 (2 mg IgG/ml gel) at 4 °C for 2 h. After washing the gel extensively, the antigen was eluted with 0.5 M NH_4OH containing 0.1% Nonidet P-40. The eluate was dried *in vacuo* at room temperature. The sample was dissolved in lysis buffer with 0.1 M dithiothreitol, and separated by 10% SDS-PAGE. Proteins in the gel were stained with a silver stain kit (Wako Pure Chemicals). The same procedure was repeated three times with distinct samples.

Purification of the HCL-4 antigen from placenta and partial amino acid sequence analysis

Human placenta (40 g wet weight) were homogenized in 400 ml of 40 mM phosphate buffer (pH 7.3) containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 2 mM *p*-amidinophenylmethane sulfonylfluoride hydrochloride. After centrifugation (10 000 *g*, 30 min), the concentration of Nonidet P-40 was reduced by dilution to 0.3%. The supernatant was mixed with 25 ml anti-TNP-conjugated Affi-gel 10. The unbound fraction was incubated with 1 ml

HCL-4-conjugated Affi-gel 10 (2 mg IgG/ml gel) at 4 °C overnight. After washing the gel, the antigen was eluted with 0.5 M NH₄OH containing 0.1% Nonidet P-40. The eluate was dried *in vacuo* at room temperature. The sample was separated by 10% SDS-PAGE, and proteins in the gel were transblotted onto the PVDF membrane (Millipore Corp., Bedford, MA, USA) in Tris/boric acid buffer with 0.05% SDS and 20% methanol. The protein on the PVDF membrane was stained with 0.1% Coomassie Blue R-250 (Nacalai Tesque, Kyoto, Japan) in 50% methanol and the protein band was used for N-terminal amino acid sequence analysis. Sequencing was performed by TAKARA, Japan. In some experiments, the dried eluate was treated with lysyl endopeptidase (1 mg/ml, Nacalai Tesque) at 37 °C in 0.1 M citrate buffer (pH 5.0) overnight, and the protein fragments obtained were separated by 15% SDS-PAGE. They were transblotted onto PVDF membrane as described above, and N-terminal amino acid sequences of main band were analyzed. The SWISS-PROT database was used in the analysis of amino acid sequence homology.

Western blotting analysis of CL

CL of menstrual cycle (CL days 7, 8, and 9) and CL of pregnancy of 7 ($n=2$) and 12 weeks of gestation was homogenized by sonication with lysis buffer including 2 mM *p*-amidinophenyl-methane sulfonylfluoride hydrochloride, and they were centrifuged at 15 000 *g* for 20 min. Chorion from the term placenta was also dissolved. Each supernatant including 40 µg protein was electrophoresed in 10% SDS-polyacrylamide gel, and transblotted on the PVDF membrane as above in Tris/glycine buffer with 0.03% SDS and 20% methanol. The PVDF membrane was treated with Block Ace (Dainihon Pharmaceuticals, Osaka, Japan), rabbit pAb against human MAOA (H-70, Santa Cruz Biotechnology, Inc.) and human β -actin (ab8227; Abcam Co. Tokyo, Japan), and then horseradish peroxidase (HRP)-conjugated goat antibody to rabbit IgG (DAKO Japan; Honda *et al.* 1995). HRP was visualized with 3,3'-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan). The intensity of the bands was quantified and analyzed by an NIH image software program (Image/J, <http://rsb.info.nih.gov/ij/>). The data were expressed as a ratio of MAOA densitometric intensity divided by the corresponding intensity of β -actin.

RNA isolation and northern blotting

Total RNAs from human menstrual CL on CL days 7, 8 ($n=2$), and 9, and pregnant CL at 7 ($n=2$), 9, and 12 weeks of gestation were isolated using a commercial kit (TRIzol, Invitrogen). Five micrograms of total RNA from the CL were reverse transcribed with random primers using a commercial kit (First-Strand cDNA Synthesis Kit; Pharmacia, Inc). The resulting cDNA mixtures were subjected to 30 cycles of PCR amplification with oligonucleotide primers designed based on the sequence of human MAOA cDNA (sense primer 5'-AGGAAATAAGGAAGAAGA-3': positions 1170–1189; antisense primer 5'-ACACTGACAAATAAGATGGA-3': positions 1848–1867, GenBank M68840; Bach *et al.* 1988), or with human RPS26 primers (sense primer 5'-GGTCCG TGCCTCCAAGATGA-3': positions 8–27; antisense primer 5'-TAAATCGGGGTGGGGGTGTT-3': positions 308–327;

Vincent *et al.* 1993). After PCR amplification, 10 µl of each PCR product was electrophoresed on a 1.5% agarose gel, and amplified bands were detected by ethidium bromide staining. After cloning, the PCR product and verifying its sequence, the enzymatically digested cDNA insert was purified and used as a probe for subsequent northern blot analysis that was performed as previously reported (Higuchi *et al.* 1995). In brief, 10 µg total RNAs were electrophoresed on a 1.0% agarose-formaldehyde gel and then transferred to nylon membranes. The membranes were incubated with a prehybridization solution (Rapid Hyb, Amersham-Pharmacia) for 30 min at 65 °C and then hybridized with [³²P] dCTP-labeled MAOA cDNA probes in the same solution for 2 h at 65 °C. After hybridization, the filters were washed according to the manufacturer's protocol and then subjected to autoradiography. The membranes were stripped and then reprobated with RPS26 cDNA. The intensity of the bands was quantified and analyzed by an NIH image software program (Image/J). The data were expressed as a ratio of MAOA densitometric intensity divided by the corresponding intensity of RPS26.

Statistical analysis

Data are shown as means $\pm$ s.e.m. The data of bands of MAOA protein (western blot) and mRNA (northern blot) in corpora lutea of mid-luteal phase and pregnancy were analyzed by an unpaired *t*-test. The expression scores of HCL-4 antigen on large and small luteal cells in CL of menstrual cycle and CL of pregnancy were analyzed by Mann–Whitney analysis. The above statistical analyses were performed by the personal computer soft StatView J 5.0.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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