A monoclonal antibody, HCL-2, raised against human luteal cells reacts with apolipoprotein-B and detects the uptake of low density lipoprotein by luteinizing granulosa cells

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A monoclonal antibody, HCL-2, was raised by immunizing mice against human luteal cells. HCL-2 reacted with luteal cells and villous trophoblasts. The sodium dodecyl sulphate–polyacrylamide gel electrophoresis profile of immunopurified antigens from corpus luteum, chorionic villi, and placenta showed the same main protein band, the molecular mass of which is >200 kDa. The sequence of a portion of the N-terminal region of the antigenic protein purified from placenta was identical to that of apolipoprotein-B. The antigen purified from human serum and low density lipoprotein (LDL) using HCL-2 showed the same protein band as that from corpus luteum. Furthermore, the amino acid sequence (20 amino acids) of the protein purified from serum was also identical to that of apolipoprotein-B. Thus, we concluded that HCL-2 antigen is apolipoprotein-B. Human luteinizing granulosa cells isolated from the patients undergoing in-vitro fertilization treatment were cultured in the medium containing lipoprotein-deficient serum with or without supplementation of LDL. Using HCL-2, apolipoprotein-B was immunocytochemically detected on granulosa cells only in the presence of LDL. These findings showed that the uptake of LDL by granulosa cells was detected by immunocytochemical staining of apolipoprotein-B, indicating that HCL-2 is useful for analysing dynamic utilization of LDL by ovarian cells.

Key words: apolipoprotein-B/granulosa cells/HCL-2/luteal cells/monoclonal antibody

Introduction

Using monoclonal antibodies (mAb), we demonstrated that membrane-bound aminopeptidase N (EC 3.4.11.2) and dipeptidyl peptidase IV (EC 3.4.14) are differentiation-related cell surface molecules on human granulosa cells and thecal cells (Fujiwara et al., 1992a,b). The inhibition of aminopeptidases enhanced gonadotrophin-induced ovulation in vivo and steroidogenesis in vitro (Nakamura et al., 1996; Tachibana et al., 1996). We proposed a new ovarian regulatory system in which these membrane-bound peptidases are involved in folliculogenesis and luteal function by regulating extracellular peptide concentrations. To identify other differentiation antigens involved in mechanisms of ovarian cell differentiation, we raised several mAb by immunizing them against porcine or human granulosa cells (Fujiwara et al., 1993a,b, 1994a, 1995; Honda et al., 1995). For example, we reported that mAb OG-1 recognized a cell surface molecule of human granulosa cells in growing follicles and corpora lutea (CL) of early and mid-luteal phases (Fujiwara et al., 1993a). We purified the OG-1 antigen from the human placenta and partially sequenced the N-terminal amino acids, which revealed that the OG-1 antigen is identical to integrin α6 (Honda et al., 1995), and we later indicated the involvement of integrins in granulosa cell differentiation (Fujiwara et al., 1996b, 1997). Thus, we think that the analysis of differentiation antigens via mAb, or mAb production, is an attractive approach for the investigation of ovarian cell physiology.

Recently, we produced mAb by immunizing mice against human luteal cells isolated from menstrual and pregnancy CL. One of these mAb, human corpus luteum antigen-1 (HCL-1), specifically detected large luteal cells of CL in mid- to late luteal phase and those of pregnancy CL, indicating that large luteal cells in early pregnancy are derived from those in the menstrual cycle, not from small luteal cells (Fujiwara et al., 1996a). Here, we describe the other mAb, HCL-2, which reacted with a protein expressed in the human luteal cells. Our analysis of the purified antigen revealed that HCL-2 antigen is identical to apolipoprotein-B. We also examined the significance of immunohistological detection of the apolipoprotein-B by HCL-2 in steroid-producing cells using cultured luteinizing granulosa cells.

Materials and methods

Samples

Ovarian tissues were obtained from eight women, aged between 28 and 43 years old. They had undergone unilateral ovarian cystectomy or oophorectomy and contralateral wedge resection to treat benign ovarian tumours. All women had a history of regular menstrual cycles (28–30 days) and their ovulatory basal body temperature charts were of normal luteal phase length. Macroscopically and microscopically normal regions of these tissues were used for this study. Fresh human granulosa cells were obtained from patients aged from 27 to 41 years who had undergone treatment for in-vitro fertilization (IVF). First trimester human chorion samples (6–8 weeks of gestation, n = 3) were obtained from patients who had undergone legal abortion. Term placentas (n = 5) were obtained at delivery. Informed consent was obtained from all patients prior to the study.
HCL-2 detects apolipoprotein-B uptake in granulosa cells

Figure 1. HCL-2 antigen expression in corpus luteum on day 7 detected by indirect immunofluorescence staining. (A) Haematoxylin and eosin staining. (B) Staining with HCL-2 mAb. (C) Staining with anti-apolipoprotein-B mAb (MAB012). (D) Negative control stained with anti-TNP mAb. HCL-2 antigen and apolipoprotein-B were expressed on the granular-shaped structure near the nuclei in the cytoplasm of both large (LL) and small (SL) luteal cells. Both proteins were also detected along the cell membrane, showing the similar expression profiles. Original magnification ×120. Bar = 100 µm.

Monoclonal antibody production
Luteal cells were isolated as described (Fujiwara et al., 1996a). Briefly, the CL was separated from connective tissue, then minced with scissors and incubated in Roswell Park Memorial Institute (RPMI) 1640 medium (Flow Laboratories, Irvine, UK) containing 5% fetal calf serum (FCS; Flow Laboratories, McLean, VA, USA), 0.2% collagenase Type I (Sigma Chemical Co., St Louis, MO, USA), 0.2% hyaluronidase Type I-S (Sigma), and 0.005% deoxyribonuclease I (Sigma) at 37°C for 1 h. The cell suspension was overlaid on Ficoll-Hypaque (Nacalai Tesque, Kyoto, Japan) and centrifuged for 20 min at 400 g. The cells at the interphase were washed twice and resuspended in phosphate-buffered saline (PBS). Eight week old BALB/c mice were injected i.p. with 2×10⁶ human luteal cells per month over a period of 4 months. Spleen cells from the immunized mice were fused with X63Ag8 myeloma cells using polyethylene glycol 4000 5 days after the last immunization (Köhler and Milstein, 1975).

Supernatants from the growing hybridomas were screened by means of indirect immunofluorescence staining of CL cryosections, which were prepared as described (Fujiwara et al., 1996a). Briefly, fresh CL were 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 µm 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, then fixed with acetone at −20°C. The cryosections were incubated with the supernatants of the growing hybridomas for 60 min at room temperature. After washing in PBS, they were incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (diluted 1:40; Dako Japan Co. Ltd, Kyoto, Japan) for 30 min at room temperature in the dark. The slides were washed extensively, mounted with glycerin–PBS (1:1, vol/vol), and examined under a fluorescence microscope (Nikon, Tokyo, Japan).

The hybridomas producing antibodies of interest were cloned twice by limiting dilution. The immunoglobulin isotype was determined using an isotyping kit for mouse monoclonal antibodies (SeroTeck Ltd, Oxford, UK). The positive hybridoma clones were expanded and injected i.p. into female mice treated with pristane (2,6,10-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, WI, USA). The IgG fraction was purified from ascitic fluid using Affi-Gel protein A (Bio-Rad Labs, Hercules, CA, USA).

Immunohistochemical examination of HCL-2 antigen expression on CL
The five samples obtained for immunization were also used for immunohistological study. These samples contained one CL on day 4, two CL on day 6, one CL on day 7 and one CL on day 8. The CL day was determined and re-evaluated according to Corner’s definition, using haematoxylin and eosin-stained tissue sections from 10% formalin-fixed and paraffin-embedded samples. For example, the day after ovulation is CL day 2 (Corner, 1956).

Purified HCL-2 mAb (5 µg/ml, diluted in culture medium) was used for indirect immunofluorescence staining as described above. Anti-trinitrophenyl (TNP) mouse mAb (unrelated mAb, IgG1 class, 5 µg/ml) was used as the negative control (Tsujimura et al., 1990).

Purification of the HCL-2 antigen from CL, chorionic villi during early pregnancy and term placenta
Purification of the HCL-2 antigen was performed as previously reported (Fujiwara et al., 1993a). Tissue materials (CL, 0.5 g; chorionic villi, 1 g; placenta, 5 g, wet weight) were homogenized in 10 or 50 ml of 40 mM phosphate buffer, pH 7.3, containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (Sigma), 0.2 mg/ml
phenylmethylsulphonyl fluoride (Wako Pure Chemicals, Osaka, Japan), 10 µg/ml leupeptin (Peptide Institute Inc., Osaka, Japan), and 10 µg/ml pepstatin (Peptide Institute Inc.). After centrifugation (11 000 g; 30 min), the concentration of Nonidet P-40 was reduced by dilution to 0.3%. The supernatant was passed through a column containing 10 ml of anti-TNP-conjugated Affi-gel 10 (2 mg IgG/ml gel; Bio-Rad Labs, Hercules, CA, USA) at 4°C to remove non-specifically bound compounds. The through-pass fraction was incubated with 0.15 ml of HCL-2-conjugated Affigel 10 (2 mg IgG/ml gel) at 4°C for 2 h. After extensively 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 dissolved in lysis buffer containing 0.2 mg/ml phenylmethylsulphonyl fluoride hydrochloride. After centrifugation (15 000 g; 30 min), the supernatant was filtered through a 10 µm-pored membrane, and then the concentration of Nonidet P-40 was reduced by dilution to 0.3%. The sample was incubated with 20 ml of anti-TNP-conjugated Affigel 10 at 4°C for 1 h, and then the gel was removed. The lysate was incubated with 0.6 ml of HCL-2-conjugated Affigel 10 at 4°C for 2 h. The antigen was eluted as described above. This procedure was repeated seven times using three samples, and the purified antigen was dialysed in 3 mM Tris–HCl buffer, pH 6.8, containing 0.1% SDS, and was dried. The purified antigen was electrophoresed by 6% SDS–PAGE. The protein in the polyacrylamide gel was transblotted on polyvinylidene difluoride (PVDF) membrane (Millipore Co., Bedford, MA, USA) in 50 mM Tris–HCl buffer, pH 6.8, containing 0.1% SDS, and was dried. The purified antigen was electrophoresed by 6% SDS–PAGE. The protein in the polyacrylamide gel was transblotted on polyvinyldene difluoride (PVDF) membrane (Millipore Co., Bedford, MA, USA) in 50 mM Tris–HCl buffer, pH 6.8, containing 0.1% SDS, and was dried. The purified antigen was electrophoresed by 6% SDS–PAGE. The protein in the polyacrylamide gel was transblotted on polyvinylidene difluoride (PVDF) membrane (Millipore Co., Bedford, MA, USA) in 50 mM Tris, 15 mM boric acid, 0.05% SDS, and 20% ethanol. The proteins were stained with Coomassie Blue, and the main protein band was cut and analysed by amino acid sequencer PSQ-1 (Shimazu Co., Kyoto, Japan). The SWISS-PROT and GenBank data bases were used in the analysis of amino acid sequence homology.

**Purification of HCL-2 antigen from human LDL and serum**

Purified human LDL was generously supplied by Dr M. Murakami (Department of Geriatric Medicine, Kyoto University, Japan) (Goldstein et al., 1983). 200 ml of serum was mixed with 400 ml of lysis buffer (pH 7.3, 40 mM phosphate buffer containing 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40) containing 0.2 mg/ml phenylmethylsulphonyl fluoride hydrochloride. 10 mg of LDL was lysed with lysis buffer (10 ml, pH 7.3, 40 mM phosphate buffer containing 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40). Immunoadfinity chromatography and silver staining were performed as described above.

**Analysis of the partial amino acid sequence of the protein purified from placenta**

Three hundred grams of human placenta were homogenized in 800 ml of lysis buffer containing 0.2 mg/ml phenylmethylsulphonyl fluoride hydrochloride. After centrifugation (15 000 g; 30 min), the supernatant was filtered through a 10 µm-pored membrane, and then the concentration of Nonidet P-40 was reduced by dilution to 0.3%. The sample was incubated with 20 ml of anti-TNP-conjugated Affigel 10 at 4°C for 1 h, and then the gel was removed. The lysate was incubated with 0.6 ml of HCL-2-conjugated Affigel 10 at 4°C for 2 h. The antigen was eluted as described above. This procedure was repeated seven times using three samples, and the purified antigen was dialysed in 3 mM Tris–HCl buffer, pH 6.8, containing 0.1% SDS, and was dried. The purified antigen was electrophoresed by 6% SDS–PAGE. The protein in the polyacrylamide gel was transblotted on polyvinylidene difluoride (PVDF) membrane (Millipore Co., Bedford, MA, USA) in 50 mM Tris, 15 mM boric acid, 0.05% SDS, and 20% ethanol. The proteins were stained with Coomassie Blue, and the main protein band was cut and analysed by amino acid sequencer PSQ-1 (Shimazu Co., Kyoto, Japan). The SWISS-PROT and GenBank data bases were used in the analysis of amino acid sequence homology.

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Expression of HCL-2 antigen on isolated and cultured human granulosa cells

Human granulosa cells were isolated from patients who had undergone treatment for IVF as described (Fujiiwara et al., 1994b). Briefly, ovarian stimulation was achieved with a gonadotrophin-releasing hormone analogue (buserelin acetate; Hoechst Japan Co. Ltd, Tokyo, Japan) and human menopausal gonadotrophin (HMG; Organon Japan Co. Ltd, Tokyo, Japan). Follicles were aspirated 36 h after the administration of human chorionic gonadotrophin (HCG, 5000 IU, i.m.; Mochida Pharmaceutical Co. Ltd, Osaka, Japan). The suspension of granulosa cells was overlaid on Ficoll-Hypaque and centrifuged at 400 g for 30 min. The cells collected from the interphase were resuspended in RPMI 1640 medium. Viable granulosa cells (5 × 10⁴/~ 0.7 ml) suspended in culture medium supplemented with 10% lipoprotein-deficient serum (generous gift from Dr H.Yoshida, Department of Geriatric Medicine, Kyoto University) (Goldstein et al., 1983) were inoculated into each well of 4-chamber culture slides (Lab-Tec, Nunc Inc., Naperville, IL, USA). The cells were then incubated for 3 days. The next day (day 1), the medium was discarded to remove unattached cells and replaced with a fresh medium with lipoprotein-deficient serum containing HCG (1 IU/ml) in the absence or presence of LDL (10 µg/ml). On days 1 and 3, the cultured slides were washed gently three times with PBS, thoroughly dried, and fixed with acetone at –20°C.

The slides were indirectly stained using HCL-2 and anti-TNP mAb as described above. Three independent experiments were performed. Sometime after 3 day culture under HCG stimulation without LDL supplement, the granulosa cells were replaced with fresh medium containing lipoprotein-deficient serum and HCG (1 IU/ml) in the absence or presence of LDL (10 µg/ml). At 1, 5, 10, 30 min and 1 h after replacement of the medium, the cultured slides were washed and fixed by cold acetone. In the same conditions, the media were exchanged and collected for progesterone assay after 1, 3 and 6 h and the culture cells was dispersed with 0.05% trypsin and 0.05% EDTA. The number of viable granulosa cells were counted under microscopy by Trypan Blue exclusion. This procedure was independently repeated five times.

Radioimmunoassay of progesterone

The concentration of progesterone in the culture media was determined by radioimmunoassay kits (Daichi Radioisotope Laboratories, Ltd, Tokyo, Japan) as described previously (Tachibana et al., 1996). The values for progesterone secretion were normalized on the basis of the number of cells.

Statistical analysis

Progesterone production in five experiments was examined in triplicate. The data were expressed as the mean ± SEM and were analysed by the two-tailed paired t-test.

HCL-2 detects apolipoprotein-B uptake in granulosa cells

Figure 4. The N-terminal amino acid sequences of the major proteins as determined by immunoaffinity chromatography using HCL-2. The seven and 20 amino acid sequences determined from the major proteins purified from placenta and serum were identical to those of human apolipoprotein-B, respectively. –, not determined.

Results

The expression profiles of HCL-2 antigen in human CL, in chorionic villi during early pregnancy and in term placenta

One hybridoma was selected by immunohistochemistry. This monoclonal antibody was named HCL-2 and belonged to IgG1 isotype.

In corpora lutea on CL day 7, HCL-2 antigen was expressed in the cytoplasm of both large and small luteal cells (Figure 1). HCL-2 antigen was also detected in the chorionic trophoblasts in early pregnancy (Figure 2). In the term placenta, the HCL-2 antigen was very weakly expressed on the trophoblasts and stromal cells of chorionic villi (data not shown).

Purification of HCL-2 antigen from CL, from chorionic villi during early pregnancy, and from term placenta

Antigenic molecules were affinity-purified from CL, chorionic villi and placenta. SDS–PAGE profile of HCL-2 antigen purified from these tissues shared the same major protein band, the molecular mass of which is ✈>200 kDa (Figure 3).

Analysis of the partial amino acid sequence of HCL-2 antigen purified from term placenta and serum

The sequence of seven amino acids from the fourth to the 10th residues of the N-terminal region of the protein from placenta were analysed. The determined seven amino acids were identical to those in the human apolipoprotein-B (Figure 4).

SDS–PAGE profile of HCL-2 antigen from LDL and serum had the same major protein bands as those from CL and placenta (Figure 3). Therefore, we then analysed the sequence of the N-terminal region of the HCL-2 antigen purified from serum. The 20 amino acid sequence determined from the major protein in the serum was also identical to that of human apolipoprotein-B (Figure 4).

The expression of apolipoprotein-B in human ovaries detected by anti-apolipoprotein-B mAb

The expression of apolipoprotein-B in human CL was also examined with two available anti-apolipoprotein-B mAb, which were obtained from Medix Biotech Inc., Foster City, CA, USA (A016-10 mAb) and Chemicon International Inc., Temecula, CA, USA (MAB012 mAb). Apolipoprotein-B was detected in the cytoplasm near the nuclei of both large and small luteal cells and along the cell membrane. The pattern of staining of apolipoprotein-B was granular-shaped. These expression profiles of apolipoprotein-B were similar to that of HCL-2 antigen (Figure 1).

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Figure 5. Detection of HCL-2 antigen on human luteinizing granulosa cells cultured in a medium with or without low density lipoprotein (LDL) by indirect immunofluorescence staining. (A–D) Culture with LDL. (E–H) Culture without LDL. (A, C, E and G) Phase-contrast pictures. (B and F) HCL-2 antigen. (D and H) Negative controls (anti-TNP mAb). HCL-2 antigen was clearly detected in the cytoplasm of human granulosa cells (arrows) cultured in the medium containing LDL for 3 days (B), whereas it was hardly detected in those cultured without LDL (F). Original magnification ×240. Bar = 50 µm.

The expression of apolipoprotein-B and production of progesterone on cultured human luteinizing granulosa cells under the supplementation with or without LDL

Although apolipoprotein-B was detected on granulosa cells on day 1 despite the lack of LDL (data not shown), it was hardly detected on granulosa cells which were cultured in a medium without lipoproteins for 3 days. In contrast, granulosa cells cultured in the medium containing LDL clearly expressed apolipoprotein-B on day 3 (Figure 5).

Granulosa cells cultured for 3 days without lipoprotein immediately incorporated apolipoprotein-B after the medium
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Figure 6. Detection of HCL-2 antigen on human luteinizing granulosa cells after the supplementation with low density lipoprotein (LDL) by indirect immunofluorescence staining. (A and B) Culture with lipoprotein-deficient serum for 3 days. (C and D) Culture for 1 min after replacement of the medium with LDL. (E and F) Culture for 30 min after replacement of the medium with LDL. (A, C and E) Phase-contrast pictures. (B, D and F) HCL-2 antigen was not detected on human granulosa cells cultured with lipoprotein-deficient serum (B), whereas in cells supplemented with LDL it was initially detected on the cell surface region (arrows) after 1 min (D), and then detected in the cytoplasmic region (arrows) of the granulosa cells after 30 min (F). Original magnification ×240. Bar = 50 µm.

was supplemented with LDL. Apolipoprotein-B was initially detected on the cell surface region after 1 min and then detected in the cytoplasm of the granulosa cells after 30 min in the presence of LDL (Figure 6).

Within 1 h after LDL supplementation, a significant rise of progesterone production was observed in the LDL-supplemented group. This significantly high secretion of progesterone was continued throughout 6 h culture in the LDL-supplemented group as compared with non-supplemented group (Figure 7).

Discussion
In this study, we raised a mAb, named HCL-2, which recognizes an antigen localized in the cytoplasm of human luteal cells. The antigen purified from CL by immunoaffinity consists of one main protein, the molecular mass of which is >200 kDa.

The molecular mass of the major protein purified from human term placenta using HCL-2 was similar to that purified from CL. The partial amino acid sequence of HCL-2 antigen purified from placenta was identical to that of apolipoprotein-B. Apolipoprotein-B is the core protein of LDL that contains about 1500 molecules of cholesteryl ester, and is responsible for the specific binding of LDL to a cell surface receptor protein (the LDL receptor). The main proteins purified from human serum and LDL using HCL-2 showed the same molecular mass as those from CL, and the amino acid sequence of the protein from serum was identical to that of apolipoprotein-B. After binding to its receptor, LDL is imported into cytoplasm by receptor-mediated endocytosis and then apolipoprotein-B is degraded (Brown and Goldstein, 1986). The analysis of apolipoprotein-B was reportedly difficult since apolipoprotein-B can aggregate extensively via covalent and non-covalent interactions and it can be degraded by proteolysis,
binding of LDL to LDL receptor. After 30 min, intracytoplasmic supplementation of LDL, which probably showed the presence of apolipoprotein-B in luteinizing granulosa cells, as suggested by Brannian et al. (1984). While apolipoprotein-B was clearly detected in the cultured granulosa cells on day 1, the immunoreactive apolipoprotein-B become almost undetectable in the granulosa cells after 3 days of culture in a lipoprotein-deficient serum. This shows that the epitope of HCL-2 mAb is degraded within at least 3 days. Therefore, the detection of immunoreactive apolipoprotein-B by HCL-2 mAb may represent apolipoprotein-B taken up within the last few days.

In the macaque, the dynamic uptake of LDL of ovarian cells in vitro has been extensively examined using fluorescently labelled LDL (Brannian et al., 1991, 1992; Brannian and Stouffer, 1993). However, this technique cannot be applied to humans since the safety of i.v. administration of fluorescently tagged LDL is not established. The present study showed that a simple method such as immunohistochemistry using HCL-2 mAb can detect the dynamic uptake of apolipoprotein-B in human cells in vitro and that it can be also used to assess the in-vivo uptake of apolipoprotein-B by steroid hormone-producing tissues. This will contribute to analysing the human steroid hormone producing cells in various organs including the ovary in vivo and in vitro.

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