Expression of DAZL protein in the human corpus luteum

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The DAZL gene and its homologues are required for the development of male and female germ cells in different species. However, their role in other aspects of human reproduction is not known. We have generated a polyclonal antibody to the DAZL protein and developed a sensitive standard curve quantitative-competitive–RT–PCR assay to characterize the expression of DAZL in the human corpus luteum (CL). DAZL transcripts are expressed in the CL, but the concentrations decreased with advancing luteal phase. In accordance with the mRNA data, DAZL protein was most abundant in the early phase CL. Immunohistochemical staining showed DAZL protein in the cytoplasm of granulosa–luteal cells. The distinct expression pattern of DAZL protein in the human CL may play an important role in the regulation of luteal function.

Key words: corpus luteum/DAZL protein/DAZL transcript/granulosa–luteal cell

Introduction

One-sixth of human couples are infertile but otherwise healthy (Hull et al., 1985). In most infertile couples, the underlying cause cannot be identified with certainty. Recently, screening with markers on the long arm of the human Y chromosome has detected Yq microdeletions in 5–15% of males with non-obstructive azoospermia. Among cases with Yq microdeletions, a deletion involving the DAZ (deleted in azoospermia) gene family represents the most frequent finding (Reijo et al., 1995; Vought et al., 1996; Simoni et al., 1998; McElreavey and Krausz, 1999). The DAZ gene has an autosomal homologue, DAZL (DAZ-like), on chromosome 3p24. It is highly homologous to the DAZ gene, with 83% similarity in the coding region of the cDNA. Both genes encode RNA binding proteins (Saxena et al., 1996; Shan et al., 1996; Yen et al., 1996; Chai et al., 1997). It is believed that the DAZ gene arose from the transposition, repeat amplification and pruning of the autosomal gene DAZL ~40 million years ago, during primate evolution (Saxena et al., 1996).

The DAZL gene and its homologues are required for development of male and female germ cells in different species. In Caenorhabditis elegans, daz-1 is an essential factor for female meiosis but dispensable for male meiosis (Karashima et al., 2000). In Xenopus, maternal Xdazl RNA is required for early differentiation and migration of primordial germ cells (Houston and King, 2000). In Drosophila, loss of the DAZL homologue, boule, results in a block of meiotic division only in the male germ line (Eberhart et al., 1996). In mice, loss of the Dazl gene leads to a loss of germ cells in both male and females, suggesting that Dazl gene expression is necessary for gametogenesis in both sexes (Ruggiu et al., 1997). The spermatogenic defects of boule-negative flies can be partially rescued by the Xenopus Xdazl gene (Houston et al., 1988). Similarly, sterility of Dazl knock-out mice can be partially rescued by the human DAZ gene (Slee et al., 1999). These facts indicate functional conservation of DAZ, Dazl and Xdazl.

Unlike its homologue on the Y chromosome, the importance of the DAZL gene in male and female reproduction is completely unknown. In the male reproductive system, DAZL protein is expressed at multiple stages of germ cell development, including gonocytes, spermatogonia, spermatocytes, spermatids and spermatoozoa (Reijo et al., 2000; Lin et al., 2001, 2002). In the female reproductive system, the DAZL gene is expressed in fetal oogonium, adult oocytes, granulosa cells and theca interna (Dorfman et al., 1999; Nishi et al., 1999; Brekhman et al., 2000). In this study, we have provided evidence that the DAZL protein is expressed in granulosa–luteal (GL) cells from different phases of the corpus luteum (CL).

Materials and methods

Collection of ovarian tissue

We collected six samples of CL: two in early luteal phase, two in mid-luteal phase and two in late luteal phase. The specimens were taken during operations either by laparoscopic surgery for CL rupture or by laparotomy for benign gynaecological conditions. Each patient had regular menstrual periods and the dating was confirmed by either a body temperature chart or histology of endometrium. The luteal phase was subdivided into early luteal phase (day 15–19 of the cycle or young CL, 1–5 days of age), mid-luteal phase (day 20–24 of the cycle or mature CL, 6–10 days of age) and late luteal phase (day 25–27 or old CL, 11–13 days of age). Day 14 was considered as CL day 0.

Generation of anti-DAZL antibody and Western blotting

The details of antibody generation and Western blotting have been described in our previous publications (Lin et al., 2001, 2002). In brief, a peptide consisting of amino acid residues #272 to #290 (TQDDYFKDKRVHHFR-RSRA) at the COOH terminus of the DAZL protein (GenBank accession No. NP_001342) was synthesized (Sigma-Genosys, Woodlands, Texas, USA). It was conjugated to a carrier protein (Imject Maleimide Activated mCMLH kit; Pierce, Rockford, IL, USA) to be used as an immunogen. Three New Zealand white rabbits were immunized with immunogen every 2 weeks. Blood
samples were taken before immunization as a pre-immune control. The titre of antibody was determined by enzyme-linked immunosorbent assay (ELISA) and the specificity of the antibody was tested by Western blot. Serum antibodies were then affinity-purified on protein A columns according to the manufacturer’s instructions (ImmunoPure® Plus Immobilized Protein A IgG Purification Kit; Pierce).

For Western blot analysis, the tissues were homogenized and mixed with twice the volume of lysis buffer containing protease inhibitor (T-PERM® Tissue Protein Extraction Reagent; Pierce). After complete mixing, the samples were put on ice for 30 min and sonicated vigorously for 20 s to shear chromosomal DNA. Approximately 20 µg of total protein was then fractionated on a 12% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using a BioRad transfer system at 110 V for 1 h. The membranes were washed with Tris-buffered saline (TBS) containing 0.5% Tween 20, and incubated in a blocking solution (5% solution of mild powder in the wash buffer) for 1 h. The membranes were then incubated in a 1:100 to 1:2500 dilution of a primary antisera overnight and washed three times with the wash buffer, followed by incubation with a 1:10 000 dilution of the secondary antibody (goat anti-rabbit IgG, peroxidase conjugated from Pierce) in the wash buffer. The filters were then washed several times, and the peroxidase activities were visualized using the SuperSignal substrate according to the manufacturer’s instructions (Pierce). We have shown the presence of a single band of ~33.5 kDa representing DAZL protein in testicular and semen extracts (Lin et al., 2001, 2002). In the present study, pre-immune serum and antisera pre-incubated with immunogen (peptide) were used as negative controls in the Western blot analysis.

Immunohistochemical staining

The procedure for immunohistochemical staining has been previously described by our laboratory (Lin et al., 2001). Briefly, specimens were dehydrated, embedded in paraffin and sectioned at 5 µm thickness. Sections were deparaffinized with 100% xylene and rehydrated with 100, 95 and 70% ethanol. The slides were then blocked with 3% hydrogen peroxide in absolute methanol for 15 min, washed with water for 5 min, and heated at 90°C for 5 min in pre-heated 0.01 mol/l citrate buffer. After cooling, the slides were washed twice with TBS for 5 min and treated with 3% goat serum for 30 min at room temperature. The slides were incubated with primary antibody (1:1000) for 60 min at room temperature. Following the washing steps with TBS, sections were incubated with biotinylated goat anti-rabbit IgG antibody (Dako, CA, USA) for 30 min at room temperature, washed with TBS, then incubated with avidin-biotinylated peroxidase complex for 30 min at room temperature, followed by reaction with diaminobenzidin tetrachloride/hydrogen peroxide. Sections were subsequently counterstained with haematoxylin, then dehydrated and mounted.

The specificity of the antibody against DAZL protein was examined using pre-immune rabbit serum and antisera pre-incubated with immunogen (peptide). For co-localization, serial sections were obtained for parallel analysis of DAZL, tissue inhibitor of matrix metalloproteinase 1 (TIMP-1, as a marker of GL cells) (Duncan et al., 1998) and 17α-hydroxylase [a marker of theca-luteal (TL) cells] (Conley et al., 1995; Duncan et al., 1998). Anti-TIMP-1 antibody was commercially available from Biotech Pharmaceuticals (Oxford, UK). The antibody to 17-α hydroxylase was kindly provided by Professor Michael Waterman at Vanderbilt University, Nashville, TN, USA.

Total RNA isolation and cDNA synthesis

The ovary tissue was stored in liquid nitrogen using 2-methylbutane as cryoprotectant until use. Before isolation of total cellular RNA, the specimen was sliced into 10 µm-thick slices. Total cellular RNA was extracted using High Pure™ RNA Tissue Kit according to manufacturer’s protocol (Boehringer Mannheim, Indianapolis, USA). For synthesis of cDNA, 12 µl aliquots of master mixture containing 2 µl of RNA, 1 µl of oligo(T)18 primer (500 ng/µl; Gibco BRL, Grand Island, NY, USA), and 10 µl of Diethylpyrocarbonate (DEPC)-treated water were heated to 70°C for 10 min and then chilled on ice. RT was performed in 25 µl aliquots of master mixture containing 1× first strand synthesis buffer, 0.1 mol/l dithiothreitol (DTT), 10 mmol/l of each dNTP, and 200 IU of RNase H minus reverse transcriptase (Superscript® II; Gibco). The RT temperature profile was 42°C for 1 h, 75°C for 15 min and final cooling to 4°C. The cDNA was aliquoted and stored at −20°C until use.

Preparation of native and competitor RNA template

The synthesis of native and competitor RNA template for internal standards was performed according to the method described previously (Lin et al., 2001; Tsai et al., 2001). Primer sequences for DAZL and GAPDH were synthesized according to published cDNA sequences. The sequences of the primers are as follows: the forward primer for DAZL was 5'-GGAGCTCTTCTGACCTCC-3', the reverse primer was 5'-CATGTGACATGACGAG-3' and the internal primer, DAZL-IR, was 5'-CATGTGACATGACGAG-3'. The forward primer for GAPDH was 5'-TGCGGCTTCCACACCATACT-3', the reverse primer was 5'-ACACCCGTGTTGCTGTA-3' and the internal primer, GAPDH-IR, was 5'-ACACCCGTGTTGCTGTA-3'. (GenBank accession number U66126 for DAZL and m33197 for GAPDH). The internal primers were paired with the upstream primer to amplify shorter competitive fragments that also contain sequences of the reverse primers.

In the present investigation, the DAZL amplicon was a 313 bp fragment and the size of competitor was 290 bp. The sizes of GAPDH amplicon were 679 and 552 bp for native control and competitor respectively. After initial confirmation by gel electrophoresis, RT–PCR products of competitor and native control of DAZL and GAPDH genes were purified using the Concert™ Rapid PCR Purification System, (Gibco) and subcloned into a pT7Blue T-vector (Novagen, Madison, WI, USA). The inserts were confirmed using an automatic sequencer (ABI 377; PE Applied Biosystems, Foster City, CA, USA). The clones served as the native and competitor for DAZL and GAPDH respectively. Plasmids containing natives or competitors were linearized by Hind III and transcribed in vitro using T7 RNA polymerase. The transcription RNAs were precipitated twice using 0.3 mol/l sodium acetate (pH 4.5) and 2.5 volumes of 100% ethanol after removal of DNA and protein from the solution. The concentrations of RNAs were quantified by OD260 absorbance, aliquoted and stored at −80°C. Each RNA aliquot was used only once to reduce variation due to potential degradation of RNA after freezing and thawing.

Standard curve quantitative-competitive (QC)–RT–PCR

The detailed procedure of standard curve QC–RT–PCR has been described previously (Tsai and Witthank, 1996, 1998; Tsai et al., 2001). Briefly, a constant amount of competitor RNA was added into a RT master mix (50 mmol/l Tris–HCl, 75 mmol/l KCl, 3 mmol/l MgCl2, pH 8.3, 10 mmol/l DTT, 100 pmol random primer, 4 mmol/l dNTPs and 50 IU MML-V reverse transcriptase). This mix was then dispensed into 0.2 ml thin wall PCR tubes and known amounts of native RNA in 2 µl of DEPC-treated water or 2 µl of total RNA samples were added individually to each tube. The final volume of RT mix was 20 µl and RT was performed at 42°C for 60 min followed by heating to 75°C for 15 min and quick chilled to 4°C in a programmable thermocycler (PTC–100; MJ Research, Watertown, MA). RT-PCR products were separated by 15 µl of PCR mix [final concentration: 20 mmol/l Tris–HCl (pH 8.4 at 25°C), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.2 mmol/l dNTPs, 0.5 IU Taq polymerase and 0.4 µmol/l of primers]. This was subjected to 30 cycles of amplification (30 s denaturation at 95°C, 30 s annealing at 57°C and 30 s elongation at 72°C) followed by final elongation at 72°C for 5 min. PCR products (10 µl) were directly separated on 0.8 or 4% agarose gels with 1× TBE (0.09 mol/l Tris, 0.09 mol/l boric acid, 0.001 mol/l EDTA, pH 8.0) buffer at 110 V for 40 min using Mini-protein® II electrophoresis system (BioRad, Richmond, CA, USA). The gel was then stained with ethidium bromide and placed on a UV illuminator equipped with a camera connected to a computer. The gel image was analysed using Alphalager™ software (Alpha Innotech Corp, San Leandro, CA, USA). A ratio was calculated for the intensity of native versus competitor bands on each lane of the gels. The logarithmic ratio of native to competitor was plotted against the logarithmic initial amounts of native to produce the standard curve and concentrations of specific mRNA transcripts were obtained by comparison to the standard curve as previously described (Tsai and Witthank, 1996, 1998).

Results

Concentrations of mRNA encoding DAZL were greatest in early CL, less abundant in mid-cycle CL and lowest in late CL (Figure 1). On the other hand, GAPDH mRNA was expressed in all three stages of
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Figure 1. The DAZL transcripts are detectable throughout the early, mid- and late luteal phases by standard curve quantitative-competitive (QC)–RT–PCR. (A) Bands for 0.025–3.2 amol of native co-amplified with 0.125 amol of competitor for 30 cycles and separated on an 8% polyacrylamide gel. (B) A standard curve of the DAZL gene QC–RT–PCR. The log ratio of native to competitor product is plotted against the log amount of initial native added to the QC–RT–PCR. (C) RT–PCR products amplified from different stages of the corpus luteum in the presence of a constant amount of competitor. The blank control incorporated reaction mixtures without cDNA or RNA. M = marker; NC = negative control.

Figure 2. Quantitation of GAPDH transcripts throughout the early, mid- and late luteal phases by standard curve quantitative-competitive (QC)–RT–PCR. (A) Bands for 0.25–32 amol of native control co-amplified with 4 amol of competitor for 30 cycles and separated on a 5% polyacrylamide gel. (B) A standard curve of the GAPDH gene QC–RT–PCR. (C) RT–PCR products amplified from different stages of the corpus luteum in the presence of a constant amount of competitor. The blank control incorporated reaction mixtures without cDNA or RNA. M = marker; NC = negative control.

Figure 3. (A) Western blot of testis and the early, mid-cycle and late corpus luteum. A protein of ~33.5 kDa is detected. (B) The bands are inhibited by pre-incubation of antiserum with the immunogen. (C) A monoclonal antibody to β-actin was used as positive control in Western blot. Were also TIMP-1-positive (data not shown). Parallel sections of mid-cycle CL using pre-absorbed antiserum and pre-immune serum did not show positive staining (Figure 5D and E respectively).

Discussion

The CL arises from reorganization of the dominant follicle after its ovulation. It is the major source of steroidogenesis in the ovary during the postovulatory phase of the cycle. The hormone-producing cells of the CL are composed of at least two distinct cell types, GL and TL cells. Besides luteal cells, non-steroid-producing cells such as...
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Figure 4. Expression of DAZL protein in the corpus luteum during the early luteal phase. (A) Immunohistochemical stain with a DAZL antibody. Two well-defined lobes in which granulosa–luteal (GL) cells (arrows) are positively stained. (B) Parallel section staining with TIMP-1 antibody. The positively stained areas (arrows) with TIMP-1 are compatible with those in A. (C) Negative control using pre-immune antiserum. Photographs are representative of six samples of early luteal phase corpus luteum. Original magnification ×350.

Figure 5. Expression of DAZL protein in the corpus luteum during the mid- and late luteal phases. (A) In mid-luteal phase, granulosa–luteal (GL) cells (indicated by arrows) show expression of DAZL protein. (B) The theca–luteal cells are stained positively (indicated by arrows) for 17α-hydroxylase. (C) The cells of late luteal phase are dispersed and much smaller than those of early luteal phase, and DAZL protein is hardly detectable in this stage. (D) Parallel section (to A and B) of mid-luteal corpus luteum stained with antiserum pre-incubated with the immunogen showing no signals. (E) Parallel section of mid-phase corpus luteum stained with pre-immune serum showing no signals. Photographs are representative of five samples of mid-luteal phase and five samples of late luteal phase corpus luteum. Original magnification ×350.

As endothelial cells, leukocytes and fibroblasts are also present in the CL. In the present study, we have showed expression of DAZL protein in GL cells. In addition, both DAZL mRNA and protein appear to be most abundant in the early luteal phase. In female mice, DazL protein is present in follicular cells and oocytes (Ruggiu et al., 1997). In humans, DAZL protein is expressed in germ cells of fetal
and adult ovaries (Tsai et al., 2000). Nishi et al. have also shown that DAZL protein is expressed in the theca interna of the maturing follicle, but not in the ovary of menopausal women (Nishi et al., 1999). The CL is the final form of a developing follicle and is the major endocrine component of the ovary to maintain early successful pregnancy (Eppig et al., 1997; Moor et al., 1998; Trounson et al., 1998). Expression of DAZL protein throughout different stages of the ovarian cycle and in both germ cell and somatic cell compartments implies important functional roles in the regulation of female reproduction.

The CL attains maturation ~5 days after ovulation and rapidly declines 9–14 days after ovulation. During normal luteolysis, two closely related events occur. First, there is loss of the capacity to synthesize and secrete progesterone and estradiol (Mcguire et al., 1994), followed by loss of the cells that comprise the CL (Pate, 1994). The dynamics of luteal cell populations during the cycle are not fully understood. Proliferating cells are most abundant on days 1–5 post-ovulation (days 15–16 of the ovarian cycle) and there is a significant decrease in proliferating cells thereafter (Gaytan et al., 1998). Most proliferating cells are of stromal origin, and most likely of vascular origin. However, only ~5% of GL cells and ~15% of TL cells proliferate during the early and mid-luteal phases (Gaytan et al., 1998). Regression of the CL is associated with both a decrease in the number of proliferating cells and an increase in the number of apoptotic cells, which are highly increased on days 25–27 of the cycle (Gaytan et al., 1998). In Drosophila, boule controls twine (cdc 25) translation and is essential in the regulation of the G2 to M phase transition during meiosis (Maines and Wasserman, 1999). Given the expression of DAZL gene in gonocytes, spermatogonia and primary spermatocytes, the protein may be involved in proliferation and meiotic division of male germ cells.

If the DAZL gene is indeed a cell cycle regulator in humans, it would be tempting to hypothesize that DAZL protein could be a cell cycle regulator in somatic cells (granulosa cells and luteal cells). However, we are inclined to hypothesize that DAZL protein in luteal cells might indicate functions other than cell-cycle regulation. Instead, we would be tempting to hypothesize that DAZL protein could be a cell cycle regulator in somatic cells (granulosa cells and luteal cells). However, we are inclined to hypothesize that DAZL protein in luteal cells might indicate functions other than cell-cycle regulation. Instead, we would be tempted to hypothesize that DAZL protein could be a cell cycle regulator in somatic cells (granulosa cells and luteal cells).

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