Size distribution of luteal cells from pregnant and non-pregnant marmoset monkeys and a comparison of the morphology of marmoset luteal cells with those from the human corpus luteum

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Summary. The size distribution of marmoset luteal cells was determined on Days 6, 14 and 20 after ovulation in non-pregnant cycles and in early pregnancy. Image analysis was used to estimate the cell diameter of dispersed cells prepared from the marmoset corpus luteum (CL). Steroidogenic cells showed a size distribution consistent with one population of cells. There was a significant increase in mean cell diameter ($P < 0.05$) from Day 6 to Day 14 in pregnant and non-pregnant animals with no further increase on Day 20. Micrographs of marmoset luteal tissue showed cells of $> 10 \mu\text{m}$ containing the organelles typical of steroid-producing cells, and smaller non-steroidogenic cells surrounding the steroid-producing cells. On the basis of microscopy, there were no areas within the CL where cell composition was noticeably different. In contrast, micrographs of human luteal tissue showed two types of steroidogenic cell; most cells were similar to those in the marmoset CL but a smaller population of smaller cells could be distinguished around the periphery and along vascular septa. It is likely that these smaller and larger types of steroidogenic cells are of theca and granulosa cell origin respectively, the two cell populations differing in the degree of electron density and amount of rough endoplasmic reticulum. A distinguishing feature between marmoset and human luteal cells was the shape of the mitochondrion which were considerably rounder in marmoset luteal cells. The origin of steroidogenic cells in the marmoset CL is unclear, although in marmosets and man the luteal cell types display morphological characteristics distinct from the large and small luteal cells described for CL of the domestic ungulates.

Keywords: luteal cell; size distribution; morphology; marmoset monkey; man

Introduction

Two distinct populations of steroidogenic cells have been described in the corpus luteum (CL) of several species, including the cow (Koos & Hansel, 1981), sheep (O'Shea *et al.*, 1979) and rabbit (Hoyer *et al.*, 1986). These populations have been distinguished by size into small and large luteal cells (Rodgers *et al.*, 1984; O'Shea *et al.*, 1989; Farin *et al.*, 1989), and further characterized by morphological and functional differences. Large cells produce a higher basal concentration of progesterone whereas small cells are more responsive to stimulation by LH/hCG. In addition, large

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cells have been shown to contain more receptors for prostaglandin (PG) F-2 α and PGE-2 (Fitz *et al.*, 1982; Alila *et al.*, 1988). The small cells are thought to be derived from theca cells in the follicle whereas the large cells are considered to be of granulosa cell origin (Alila & Hansel, 1984). Morphological studies of bovine and ovine CL have shown that the two cell types are intermingled within the CL (Mossman & Duke, 1973; O'Shea, 1987).

The characteristics of cells in the primate corpus luteum have been less widely investigated. Cell types differing in morphological characteristics have been distinguished in the rhesus monkey CL but functional differences were not described (Gulyas *et al.*, 1979). Early ultrastructural and morphological studies of human CL indicated the existence of two morphologically distinct cell types; large luteal cells (presumed to be granulosa-derived) occupying much of the CL and smaller luteal cells (presumed to be theca-derived) forming a separate layer around the periphery with small indentations into the centre (Adams & Hertig, 1969; Gillim *et al.*, 1969; Crisp *et al.*, 1970; Clements, 1987). Functional differences for human luteal cells have been reported, with small and large cells showing differences in responses similar to those described for domestic ungulates (Ohara *et al.*, 1987).

To improve understanding of the control of luteolysis and CL rescue in early pregnancy in the primate it is of importance to determine the cell characteristics and composition of the CL. Studies of cows and sheep have shown changes in luteal cell sizes and functions with cycle stage and in response to gonadotrophin treatment (Farin *et al.*, 1986, 1988; Schwall *et al.*, 1986; Harrison *et al.*, 1987). A change occurs in the progesterone response of dispersed marmoset luteal cells to hCG and the PGF-2 α analogue, cloprostenol, at different times in the cycle and in pregnancy (Webley *et al.*, 1989a). Luteal cells from non-pregnant animals on Day 14 showed a greater response to treatment than did luteal cells from animals on Day 6 and from pregnant animals on Day 14. We also established a difference in the degree of response shown by luteal cells taken from the marmoset monkey and from women. To understand these differences the aims of the present study were to determine the cell size distribution and ultrastructure of marmoset luteal cells, to establish whether any changes in cell composition occur during the cycle or in early pregnancy and to compare the structure of the marmoset CL with that of the human.

Materials and Methods

Material. Adult female marmoset monkeys were from the self-sustaining breeding colony maintained at the Institute of Zoology as described previously (Hearn, 1983). The start of the ovarian cycle was controlled by administration of a single 0.5 μ g i.m. injection of cloprostenol (Summers *et al.*, 1985) purchased as Estrumate (ICI Pharmaceuticals Division, Macclesfield, Cheshire, UK). The animals were not given any treatment to stimulate follicular development, but to synchronize ovulation an i.m. injection of hCG (75 i.u.; Chorulon: Intervet Ltd, Cambridge, UK) was administered after 8 days according to the method of Hodges *et al.* (1987). Two or three follicles ovulated in each animal. The day of ovulation (Day 0) was defined as the day preceding the rise of plasma concentrations of progesterone > 32 nmol/l (Harlow *et al.*, 1983) as measured by an enzymeimmunoassay (Hodges *et al.*, 1988).

The ovarian cycle in the marmoset is 28 days with an 8–9-day follicular phase and a 19–20-day luteal phase (Harlow *et al.*, 1983). Implantation starts on Days 11–12 after ovulation (Moore *et al.*, 1985). Luteal tissue was obtained, by laparotomy (Webley *et al.*, 1989a), from pregnant animals on Days 6, 14 or 20 after ovulation and from non-pregnant animals on Days 6 or 14 after ovulation (total N = 15; N = 3 at each stage). Non-pregnant animals were removed from their male partners 4–5 days after administration of cloprostenol and housed alone until 2–3 days after ovulation. Pregnancy was confirmed by flushing embryos or embryonic tissue from the uterus at the time of laparotomy (Hearn *et al.*, 1988).

Human luteal tissue was obtained from 5 women aged between 32 and 40 years who were undergoing laparotomy for hysterectomy. The stage of the menstrual cycle was assessed from the menstrual history and tissue was taken from women in the mid-luteal phase (Days 19–23 after the first day of the last menstrual period).

Tissue preparation. The method of preparation of dispersed luteal cells was as described previously (Webley *et al.*, 1989a). In brief, tissue from 2–3 CL from each marmoset was pooled, cut into small fragments and subjected to collagenase (Boehringer Mannheim, Lewes, Sussex, UK) dispersion (10 mg/10 ml medium) for 2 periods of 25 min. The cells were pooled in culture medium (Dulbecco's modified Eagle's medium (Flow Laboratories) containing glutamine (0.584 g/l), sodium bicarbonate (3.7 g/l) and bovine serum albumin (5 g/l)) containing deoxyribonuclease and washed a further 2 times in culture medium alone.

The cells were resuspended in 1 ml culture medium and counted in a haemocytometer. To estimate the percentage recovery of cells after dispersion a piece of weighed luteal tissue and an aliquant of dispersed cells, taken from the same, previously weighed, CL ($n = 3$ CL from different animals) were measured for DNA content using the method of Labarca & Paigen (1980).

The cells were stained for 3β -hydroxysteroid dehydrogenase according to the method of Aldred & Cooke (1983). Luteal cells (5000–10 000) in 100 μ l were frozen overnight with 100 μ l 6% dextran solution in phosphate-buffered saline (PBS), to increase the permeability of the cells. The cells were thawed and centrifuged at a minimum speed for 5 min to maintain the integrity of the cells. After removing the supernatant 100 μ l of the following mixed reagent were added: 100 μ l 5 α -androstane- 3β -ol-17-one (epiandrosterone) in dimethylformamide (2 mg/ml), 1.0 ml nitroblue tetrazolium in PBS, pH 7.4 (1 mg/ml), 700 μ l nicotinamide in PBS (1.6 mg/ml), 800 μ l NAD⁺-free salt in PBS (3 mg/ml) and 4.0 ml PBS. The cells were incubated with the reagent for 60 min at 37°C; cells were also incubated with reagent without epiandrosterone as a control. Those cells which stained blue were considered to be steroid-producing.

For electron microscopy, small pieces of marmoset and human luteal tissue (approximately 1–2 mm³) from central and peripheral regions of the CL were fixed in 2% phosphate-buffered glutaraldehyde solution for a minimum of 2 h. The tissue was post-fixed, also for a minimum of 2 h, in 1% phosphate-buffered osmium solution. Some human tissue was also fixed in 2% glutaraldehyde, 2% paraformaldehyde and post-fixed in 2% osmium, both in cacodylate buffer (0.1 mol/l; pH 7.4). Tissue was dehydrated in graded ethanol starting with 70% ethanol and embedded, *via* propylene oxide, in epon/araldite resin (Mollenhauer, 1964; Smith *et al.*, 1984). Semithin sections (1.5–2.0 μ m) were stained with 1% toluidine blue for light microscopy and suitable areas selected for electron microscopy. Ultrathin sections (0.05–0.07 μ m) were cut on a Reichert Ultracut E Ultramicrotome using a diamond knife. The sections were double stained (Stempak & Ward, 1964) with 7.5% uranyl acetate in 50% methanol and lead citrate pH 12 (Reynolds, 1963), and viewed in a Jeol 1200 E electron microscope.

For light microscopy of serial sections, CL tissue collected on Day 3 after ovulation was snap-frozen in liquid nitrogen. Serial 7- μ m frozen sections were cut, on a Slee Cryostat, through the entire CL and stored frozen at –20°C. Every 5th section was air-dried, fixed in 2% glutaraldehyde for 5 min, stained with Mayer's haemalum, dehydrated, cleared and mounted in DPX.

Determination of cell size. The method of determination of cell size was initially validated on luteal tissue previously shown to consist of 2 populations of steroidogenic cells. Bovine CL, judged to be mid-cycle, were collected fresh from the abattoir, subjected to collagenase dispersion (O'Shaughnessy & Wathes, 1988) and stained for 3β -hydroxysteroid dehydrogenase. The size distributions of dissociated cells which stained positively for 3β -hydroxysteroid dehydrogenase were determined by directly measuring the area of the cells on a microscope slide by image analysis. The image analyser, a 'System 3' purchased from Analytical Measuring Systems (Saffron Waldon, Essex, UK), was connected to a microscope *via* a video camera. Using the image edit the boundary of each cell was delineated and the area measured; the diameter was then calculated assuming the cells were circular in shape. The precision of the image analyser was calculated by measuring the diameter of 3 cells repeatedly ($n = 10$). The coefficient of variation was <5% for the 3 groups of data. The sample size required to give ± 2 s.d. of the true mean was calculated from the equation $n = 4\sigma^2/L^2$ (Snedecor & Cochran, 1956) where σ is the standard deviation and L the allowable error in the system taken as 5%. In the preparation of dispersed bovine cells 300 steroidogenic cells were measured and 100 in each of the marmoset luteal cell preparations. The cell sizes are expressed graphically as number of cells at each diameter and as a mean ± 2 s.d. Analysis of variance was used to determine statistical significance between cell sizes measured at different times in the cycle and pregnancy.

In addition to measuring the size of 100 dispersed luteal cells from each marmoset, luteal cell sizes were also measured in intact tissue, using electron micrographs and by taking the mean of 10 diameter measurements for an individual cell. Mean diameters of approximately 25 luteal cells were measured in this way for each sample. The cells chosen were those for which the morphology, judged by the bilaminar appearance of the nuclear membrane, indicated section through as near an equatorial plane as possible.

Results

The cell size distribution of bovine luteal cells taken in the mid-luteal phase is shown in Fig. 1. Two populations of steroidogenic cells could be distinguished, smaller cells of between 10 and 20 μ m in diameter and a smaller population of larger cells with a diameter of 20–33 μ m. The cell size of steroidogenic cells from the marmoset CL showed more uniformity and, while there was a spread in cell size from 14 to 32 μ m, 2 distinct populations of cells could not be distinguished. This was evident with cells measured on both Day 6 and Day 14 after ovulation (Fig. 2). Large numbers of smaller non-steroidogenic cells (<10 μ m) could be seen in the dissociated cell preparations. The percentage recovery of cells from the CL after collagenase dispersion was $18.3 \pm 6.4\%$ ($n = 3$).

-Comparison of luteal cell sizes from marmoset monkeys at various times in the cycle and pregnancy (Table 1) showed a significant increase in diameter ($P < 0.05$) from Day 6 to Day 14 in pregnant and non-pregnant animals. There was no further increase in the diameter of luteal cells

Fig. 1. Cell size (diameter μm) distribution of dispersed bovine luteal cells ($n = 300$).

Fig. 2. Cell size (diameter μm) distribution of dispersed marmoset luteal cells on (a) Day 6 after ovulation ($n = 100$) and (b) Day 14 after ovulation ($n = 100$).

from pregnant animals on Day 20. Similar cell diameters were recorded for luteal cells from pregnant and non-pregnant animals on Days 6 and 14 after ovulation. The apparent cell diameters of luteal cells from electron micrographs of intact tissue were consistent with the measured diameter of dispersed cells, with many cells having a cell diameter in the range of 17–25 μm and the cells tending to have a larger diameter on Day 14 than on Day 6 after ovulation. Again similar values were recorded for pregnant and non-pregnant animals.

All the steroidogenic cells in the marmoset CL contained the organelles typical of steroid-producing cells (Fig. 3). The light micrograph in Fig. 3(a) shows a section of a marmoset CL on Day 6 after ovulation and was typical of all the sections taken through this CL. While some inflow of cells accompanying the vascular septa which move into the centre of the CL were apparent, all the steroidogenic cells were uniform in appearance with morphology as shown in Fig. 3(b). There were no areas where cell composition was noticeably different. The luteal cells were generally rounded in shape, with round or oval nuclei showing a fine margin of heterochromatin peripherally and a prominent nucleolus. The cells contained abundant smooth endoplasmic reticulum with a vesicular appearance and sparse short profiles of rough endoplasmic reticulum (Fig. 3b). The Golgi

Table 1. Diameters (μm) of dispersed steroidogenic cells from the marmoset CL

	Day after ovulation		
	Day 6	Day 14	Day 20
Non-pregnant	17.8 ± 3.2	29.6 ± 4.8	
	19.1 ± 5.0	23.3 ± 3.6	
	20.6 ± 5.2	22.9 ± 6.2	
Mean	19.2 ± 0.8	25.3 ± 2.1	
Pregnant	18.5 ± 2.8	23.9 ± 6.2	25.8 ± 5.8
	18.8 ± 3.8	25.3 ± 7.2	22.3 ± 7.8
	16.4 ± 5.6	23.6 ± 7.6	20.3 ± 5.8
Mean	17.9 ± 0.7	24.3 ± 0.5	22.8 ± 1.6

Values are mean $\pm$ s.e.m. for 100 cells from CL of 3 individual animals (mean $\pm$ 2 s.d.) on each day.

apparatus was in the peri-nuclear region. Abundant mitochondria had tubular cristae, electron dense matrix (Fig. 3c) and generally formed a 'C' configuration around the nucleus. While the mitochondria tended to vary in shape in different cells, from predominantly round to more oval or irregular, a difference in shape was not related to any other feature of the cell. The only difference that could be distinguished between the cells was a variation, from dense to lucent, in electron density. All the cells contained lipid droplets and membrane-bound secretory vesicles (Fig. 3c). The cell membranes of adjacent luteal cells displayed considerable interdigitation and coated pits were evident at intervals. No desmosomes were found adjoining cells. Interspersed between the steroidogenic cells abundant small non-steroidogenic cells (fibroblasts) and capillaries could be distinguished.

In the human CL two cell types could be distinguished (Fig. 4a). Most of the cells were similar in size and morphology (Figs 4a, b) to those in the marmoset CL but areas of smaller, more darkly stained steroidogenic cells were evident forming small pockets around the periphery and along the vascular septa (Fig. 4a). Electron micrographs of human luteal tissue, from a portion of the CL at a distance from the pockets of smaller cells, showed round, electron-lucent cells similar to those in the marmoset CL but with more abundant lipid droplets and with mitochondria which were thinner, elongated and more irregular in shape (Figs 4b, c). The smaller, less numerous luteal cells were more elongated with oval nuclei containing small clumps of heterochromatin. The electron-dense cytoplasm contained sausage-shaped mitochondria and large non-staining inclusions which are presumed to be lipid droplets from which much of the lipid had been lost during processing of the tissue for electron microscopy (Figs 4d, e).

Discussion

The method of cell dissociation and image analysis demonstrated, in agreement with previous studies (Koos & Hansel, 1981), that luteal cells from the bovine CL consist of 2 populations of steroidogenic cells. In contrast, using the same methodology, the marmoset CL did not contain 2 distinct populations of luteal cell sizes. This was evident for dissociated cells and intact tissue, indicating that a cell type was not lost during the process of cell dissociation. In electron micrographs of marmoset CL tissue all the cells of $> 10 \mu\text{m}$ in diameter had the characteristics of steroid production and the only distinguishing feature was the degree of electron density.

Light micrographs of marmoset luteal tissue showed that the CL consisted of steroidogenic cells which were uniform in morphology, interspersed with small non-steroidogenic cells, and there were

no clear areas in which the cell composition was different. This is apparently in contrast to the human CL, as shown in the present study and in agreement with previous reports (see 'Introduction'), in which large and small steroidogenic cells could be distinguished, with the small luteal cells forming discrete areas around the periphery of the CL and along the vascular septa. That the small cells are of theca origin and the large cells derived from granulosa cells in the human CL was supported by Sasano *et al.* (1989), using immunohistochemical localization of antibodies to cytochrome P-450 for aromatase and 17 α -hydroxylase, enzymes present only in granulosa and theca cells, respectively.

The marmoset and human luteal cells differed in the shape of their mitochondria. Those in the marmoset luteal cells were generally very rounded in appearance whereas those in the human CL were long and thin. It is possible that these differences in mitochondrial shape may reflect differences in the production of progesterone; concentrations of plasma progesterone are 5–10 times higher in the marmoset than in women. A transition from elongated mitochondria with lamelliform cristae to tubular cristae occurs during luteinization and is associated with the increased production of progesterone after ovulation (Farkash *et al.*, 1986).

The origin of the cells in the marmoset CL is as yet uncertain. Marmoset luteal cells were similar in size and morphology to those human luteal cells presumed to be from the granulosa cell-derived portion of the CL, although they differed in their variation in electron density. If marmoset luteal cells are of granulosa cell origin then they may be considered equivalent to the large luteal cells in domestic ungulates. However, they were not a minority population as described for the large cells in the bovine CL (Alila *et al.*, 1988). Furthermore, they are unlikely to contain significant oxytocin as described for the ovine large luteal cell (Theodosis *et al.*, 1986) as this peptide has not been found in the marmoset CL (Auletta *et al.*, 1988) and the marmoset luteal cells did not contain an abundance of rough endoplasmic reticulum. If the marmoset luteal cells are predominantly of granulosa cell origin, then they are quite distinct from the large cells found in ovine and bovine CL. This may also apply to the larger granulosa-derived cells in the human CL which formed the majority of the luteal cell population and contained considerably less rough endoplasmic reticulum than the small luteal cells. The marmoset CL may be similar to that of the rat in which both theca and granulosa cells are incorporated into the CL although they are indistinguishable in size and morphology (Long, 1973).

The mean diameter of marmoset luteal cells increased from Day 6 to Day 14 after ovulation in pregnant and non-pregnant animals. An increase in mean luteal cell size has been reported for sheep (Schwall *et al.*, 1986; Farin *et al.*, 1986) during the oestrous cycle. Development of the ovine CL was associated with both an increase in size and number of luteal cells, with small luteal cells increasing in number and large luteal cells increasing in size. It has also been shown that treatment of ewes with LH results in the conversion of small luteal cells to large cells (Farin *et al.*, 1988). This general increase in cell size with development of the CL, perhaps in response to LH, can be seen during luteinization of human granulosa cells which increase in size from 10–14 μ m to a mean value of approximately 21 μ m for mid-luteal cells from the granulosa-derived portion of the human CL (Webley *et al.*, 1989b).

Fig. 3. Structure of the marmoset CL. **(a)** Light micrograph of CL on Day 6 after ovulation, showing an apparent uniformity of steroidogenic cells (S); on the left, the ovarian capsule (C) can be seen with a vascular septum (v). $\times 30$. **(b)** Electron micrograph of a typical steroidogenic cell, showing rounded shape with a round nucleus (N). The electron-lucent cytoplasm contains lipid droplets (L), round or oval mitochondria (M) with electron dense matrix in a C configuration around the nucleus. $\times 3500$. **(c)** Higher magnification electron micrograph of the luteal cell cytoplasm illustrating the abundance of smooth endoplasmic reticulum (S), mitochondria (M) with electron-dense matrix, lipid droplets (L) and secretory granules (s). Note the paucity of rough endoplasmic reticulum in these cells. $\times 20\,000$.

We previously described the size of human luteal cells in the mid-luteal phase (Webley *et al.*, 1989b) as being similar in range and mean values to those in the marmoset CL on Day 14. Although we and others have described large and small luteal cells in the intact human CL there are no other reports of the size distribution of dispersed cells. Ohara *et al.* (1987) separated cells of $< 20 \mu\text{m}$ as small cells and $> 20 \mu\text{m}$ as large but give no indication of the cell distribution which may indeed have formed a continuum of cell sizes rather than 2 distinct populations in agreement with our results. In the ferret, although large and small cells can be distinguished, and cover a wide range in sizes, the dissociated cells form only one population of cell size (Joseph & Mead, 1988). On the other hand, a recent study of the rhesus monkey, in which dissociated cell sizes were distinguished by flow cytometry (Hild-Petito *et al.*, 1989), demonstrated 3 populations of steroidogenic cell sizes.

The apparent uniformity of steroidogenic cells in the marmoset CL makes separation and determination of different cell responses a problem. While separation of human luteal cells at $20 \mu\text{m}$ gave a population of large cells which had a higher basal production of progesterone and lower response to hCG than small cells ($< 20 \mu\text{m}$) (Ohara *et al.*, 1987), the rationale for separating cells at $20 \mu\text{m}$ is unclear. Also at odds with the present study was the finding by Ohara *et al.* (1987) that the large cells, presumably of granulosa cell origin, were a smaller percentage of the cell population than the small cells. Although the different steroidogenic cell populations of the rhesus monkey produced different basal concentrations of progesterone, all the cells were equally responsive to stimulation by hCG and PGE-2 (Hild-Petito *et al.*, 1989). The establishment of any functional variation between marmoset luteal cells awaits the determination of cell origins and any distinguishing features between the cells which may allow their separation.

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Fig. 4. Structure of the human CL. **(a)** Light micrograph of CL from the mid-luteal phase, showing the majority of the steroidogenic cells to be pale and round in shape (P) with a smaller population of small dark cells (D) at the periphery and along the vascular septum (v). The ovarian capsule (C) can be seen on the left of the figure. $\times 30$. **(b)** Electron micrograph of the more abundant larger luteal cell showing its round shape and round or slightly irregular nuclear outline. The electron-lucent cytoplasm contains scattered lipid droplets (L), tubular shaped mitochondria (M) with electron dense matrix and abundant smooth endoplasmic reticulum (S). $\times 3500$. **(c)** Higher magnification electron micrograph of the larger luteal cell cytoplasm illustrating the elongated shape and electron-dense matrix of the mitochondria (M), and abundant vesicular smooth endoplasmic reticulum (S). Note the paucity of rough endoplasmic reticulum in these cells. $\times 20\,000$. **(d)** Electron micrograph of the smaller luteal cells showing a more oval or elongated shape with oval nucleus containing small clumps of heterochromatin. The electron-dense cytoplasm contains large lipid (L) droplets (lipid lost during processing of tissue for electron microscopy), sausage-shaped mitochondria (M) and rough endoplasmic reticulum (R). $\times 3500$. **(e)** Higher magnification electron micrograph of cytoplasm from the smaller luteal cell, illustrating the mitochondria (M) with electron-lucent matrix and profiles of rough endoplasmic reticulum (R). Note the paucity of smooth endoplasmic reticulum in these cells. $\times 20\,000$.

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