Morphology and function of human Leydig cells *in vitro*. Immunocytochemical and radioimmunological analyses

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The aim of our study was to show whether the cells isolated from testes of patients underwent bilateral orchiectomy for prostatic cancer are able to grown *in vitro*, and if so, are functionally active. Immuncytochemistry was performed to show the functional status of human cultured cells. In detail, immunolocalization of luteinizing hormone receptors (LHR), mitochondria, and cytoskeletal elements was demonstrated. Moreover, radioimmunological assay was used to measure testosterone secretion by cultured Leydig cells. Using Nomarski interference contrast and fine immunofluorescence analysis the positive immunostaining for LHR was observed in almost all Leydig cells, however it was of various intensity in individual cells. Testosterone measurement revealed significant difference between testosterone secretion by hCG-stimulated and unstimulated Leydig cells ($p<0.05$). Moreover, testosterone levels were significantly higher in 24- and 48-hour-cultures than in those of 72 hrs ($p<0.05$). Morphological analysis of Leydig cells in culture revealed the presence of mononuclear and multinucleate cells. The latter cells occurred in both hCG-stimulated and unstimulated cultures. In Leydig cells labeled with a molecular marker MitoTtracker, an abundance of mitochondria and typical distribution of microtubules and microfilaments were observed irrespective of the number of nuclei within the cell, suggesting no functional differences between mono- and multinucleate human Leydig cells *in vitro*. Since the percentage of multinucleate cells was similar in both hCG-stimulated and unstimulated cultures (23.70% and 22.80%), respectively, the appearance of these cell population seems to be independent of hormonal stimulation.

Key words: human Leydig cells, LH receptors, primary culture, hCG-stimulation, immunocytochemistry, testosterone secretion; multinucleate cells, multicolor staining.

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It is well established that testosterone biosynthesis depends on the existence of mature Leydig cells in the testicular interstitium. Human Leydig cells arise from mesenchymal cells or fibroblast-like precursor cells through a hormonally regulated differentiation process (Chemes, 1996). Production of testosterone in human and mammalian Leydig cells is dependent on LH stimulation *in vivo* and on LH/hCG stimulation in *in vitro* conditions; to respond to hormonal regulation the cells are equipped with functional receptors for LH (Amador and Bartke, 1987; Simpson *et al.*, 1987; Mendis-Handagama *et al.*, 1990; Cooke, 1996; Ramadoss *et al.*, 2006). In man, the Δ^5-metabolic pathway is the major pathway for the metabolism of pregnenolone to testosterone (Rommerts, 1990). According to Hammar and Petersson (1986) in human testis from young and elderly men with prostatic carcinoma also the 5-ene pathway is preferred. For optimal steroidogenic function a number of neuroendocrine and neuronal markers have been demonstrated in human Leydig cells *in vivo* by the group of Holstein (Middendorff *et al.*, 1993; 1995). Moreover, production of testosterone in Leydig cells, requires the presence of functionally active enzymes acting within mitochondria and the smooth endoplasmic reticulum (Payne and O'Shaughnessy, 1996; for review see Haider, 2004).

Recent studies have shown that Leydig cells become hypofunctional with age. In the rat, aged Leydig cells produce less testosterone than Leydig cells from young adult rats (Luo *et al.*, 1996; for review Zirkin *et al.*, 1997). A detailed characteristics of aged rat Leydig cells *in vivo*, including reduced testosterone biosynthesis and reduced cell volume has been described by Ewing and Zirkin (1983). Now, there is evidence from *in vitro* studies that reactive oxygen species can result in the inhibition of testosterone production in mouse Leydig cells by affecting steroidogenic enzymes (Stocco *et*

al. 1993; Peltola *et al*., 1996; Cao *et al*., 2004).

Considering human samples as a very rare and valuable biological material, the aim of this study was to show whether Leydig cells obtained from testes of elderly patients who underwent orchiectomy for prostatic cancer are able to grown *in vitro*, and if so, are functionally active. For this purpose localization of luteinizing hormone receptors (LHR) and visualization of mitochondria and cytoskeletal elements in both hCG-stimulated and unstimulated Leydig cell cultures were performed, as well as testosterone secretion by cultured Leydig cells was measured. It is worth noting that the effect of LH and an involvement of cytoskeletal proteins in steroidogenesis of mouse Leydig cells *in vitro* have been demonstrated by our own (Bilinska, 1989) and mitochondria have been described as integrally involved in Leydig cell steroidogenesis (Bilinska 1994; Kotula-Balak *et al*., 2001).

Materials and Methods

Human material

Human testes were removed from patients (n=4) undergoing bilateral orchiectomy for prostatic cancer (The Urology Clinic, Collegium Medicum, Jagiellonian University). The patients ranged in age from 60 to 67 years; they were physiologically normal. The Regional Commission of Bioethics at the Jagiellonian University approved the study; all of the patients had given their written, informed consent.

The testicular tissue was chilled immediately after surgical excision and transported on ice to the laboratory where it was set up for Leydig cell isolation and culture. All solutions were sterile and the isolation procedure was performed under aseptic conditions.

Cell isolation and culture procedure

All tissues used in this study were ascertained to be histologically normal. A piece of decapsulated testes was used for the preparation of Leydig cell suspension as described previously (Chemes *et al*., 1992) with a couple of modifications. Briefly, testicular tissue was carefully rinsed with fresh medium Ham's F-12/Dulbecco's Modified Eagle medium (DME; 1/1; v/v) supplemented with 15 mM $NaHCO_3$, 20 mM HEPES, pH 7.4; and antibiotics (penicillin, 100 U/mL; streptomycin, 10 µg/mL; nystatin,10 U/mL) and gently dissected with forceps. Then, the tissue was submitted to an enzymatic digestion in the presence of 0.04% collagenase (type I, Sigma Chemical Co. St Louis MO, USA) and soybean trypsin inhibitor (1.0 µg/mL) in F-12/DME medium, under a constant agitation at 34°C for 40 min. After three decantations the supernatant was collected, filtered through a nylon gauze and centrifuged at 200x g for 4 min. Then, the cell pellet was washed twice and kept in fresh F-12/DME medium. Next, the second collagenase treatment was performed on residual tissue fragments and all further steps of the procedure were identical as above. Finally, suspended interstitial cells obtained from the two enzymatic digestions were purified on a 4-layer discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient 21%, 26%, 34%, and 60% in F-12/DME. After centrifugation, 800 x g, for 20 min at 4°C as low temperature significantly prevents cell aggregation and then centrifugation at room temperature with low-speed, 90 x g for 10 min, the cell band between 34% and 60% Percoll containing Leydig cells was collected, washed to remove Percoll, and resuspended in Ham's F-12/DME medium (as above) supplemented additionally with 2% fetal calf serum and 10 µg/mL transferrin. The purity of the cells was about 80-83% as it was checked by a histochemical test for Δ^5, 3β-hydroxysteroid dehydrogenase (Δ^5, 3β-HSD) activity. Viability of the cells assessed by trypan blue exclusion test was <94%. Purified Leydig cells ($0.5x10^6$ cells/well) were plated in 24-well-culture dishes (Nunc, Kalmstrup, Denmark), cultured for 48 hours at 34°C in a humidified atmosphere of 5% CO_2 in air. The cells were cultured with 1.25 IU/mL hCG (human chorionic gonadotropin, Pregnyl, Organon) or without during the entire culture period. The culture media were changed every other day, collected, frozen, and stored at -20° for hormone determinations. For morphology and immunocytochemistry each well was closed with a round glass coverslip of appropriate diameter (13 mm; Menzel-Glaser, Braunschweig, Germany). At the end of culture period the coverslips were removed and the Leydig cell cultures were fixed with absolute methanol and stained with May-Grünwald Giemsa (M-GG) dyes. For immunohistochemistry the cultured cells were fixed with 2% formaldehyde freshly prepared from paraformaldehyde and permeabilized with 0.1% Triton X-100 in Tris-buffered saline (TBS; 0.05 M

Tris-HCl plus 0.15 M NaCl, pH 7.6) as described previously (Bilinska and Litwin, 1995).

To calculate the percentage of multinucleate cells in both stimulated and unstimulated Leydig cell cultures 1x100 Leydig cells in each culture were counted. Results of 4 separate measurements for each culture were expressed as mean ± SD.

Immunocytochemistry

Cultured Leydig cells were processed for the visualization of luteinizing hormone receptor (LHR) using a polyclonal rabbit antibody against LHR (1:100; BIOTREND *Chemikalien GmbH*, Köln, Germany) (Kopera *et al.*, 2008). Next, biotinylated secondary antibody, goat anti-rabbit IgG (1: 500; Vector, Burlingame CA, USA) was applied. Finally, avidin-biotinylated horseradish peroxidase complex (ABC/HRP; 1:100; Dako, Glostrup, Denmark) was used. The color reaction was developed by means of Stable DAB (Research Genetics, Inc., Huntsville AL, USA) for 3-4 min. For negative control, Leydig cell cultures were incubated in the presence of irrelevant IgGs instead of primary antibody. The cells were examined with a Leica DMR microscope (Leica Microsystems, GmBH Wetzlar, Wetzlar Germany) using Nomarski interference contrast. In some specimens fluorescein isothiocyanate (FITC)-conjugated IgG (1:80; Sigma Chemical Co. St Louis MO, USA) was applied instead of biotinylated goat anti-rabbit IgG and the cells were examined with a laser confocal microscope (LSM 510 META, Axiovert 200M, ConfoCor 3; Carl Zeiss MicroImaging GmbH, Jena Germany).

The second part of Leydig cell cultures were stained with an anti-β tubulin rabbit polyclonal antibody (1:160; Sigma Chemical Co. St Louis MO, USA) followed by FITC-conjugated IgGs as the secondary antibody (1:80; Sigma) for labeling microtubules. Additionally, some cultures were stained with rhodaminyl-phalloidin (a gift from Professor T. Wieland, Heidelberg, Germany) for labeling F-actin and DAPI (Sigma) as one of the most widely used nuclear counterstains for nuclei. (Figure 1 O-P). For selective staining of mitochondria in living Leydig cells Mito Tracker® Red CMXRos probe was used (Molecular Probes, Eugene OR, USA) (Kotula *et al.*, 2001). Then, the cells were fixed, permeabilized (as above), stained with FITC-phalloidin for F-actin (Molecular Probes) and DAPI for labeling nuclei. After each step in all the procedures, cells were carefully rinsed with Tris-buffered saline (TBS; 0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6); antibodies were also diluted in TBS buffer.

Testosterone measurement

Leydig cell culture media were analyzed for testosterone content using radioimmunological technique as described in detail (Kotula-Balak *et al.*, 2004b). It was determined using [1,2,6,7,-^{3}H]-testosterone (The Radiochemical Centre, Nycomed Amersham, Buckinghamshire, England), specific activity, 81.0 Ci/mmol, as tracer and an antibody raised in rabbit against testosterone-3-o-carboxy methylo-oxime-bovine serum albumin (BSA). The lower limit of sensitivity of the assay was in the order of 5 pg/tube. Coefficients of variation within and between assays were 7.2% and 9.6%, respectively. All samples were assayed in duplicates from 4 separate experiments. Testosterone levels are expressed in ng/10^6 cells/24h. Statistical evaluation of the data included one-way analysis of variance (with the significance level at $p<0.05$) and the Duncan's multiple range test.

Results

Morphology

On the basis of light microscopic examination all Leydig cells growing in a 72-hour-monolayer system required an initial 24-h culture period to attach to the glass (Figure 1 A-B) and next 24h in culture to form a monolayer (Figure 1 D-E). Leydig cells were either epithelioid in shape with abundant cytoplasm (Figure 1 B, D-E) or slightly elongated (Figure 1 B-C, F). Under the influence of hCG Leydig cells had a more regular multilateral shape (Figure 1 D-F). The cells from unstimulated cultures became fibroblast-like after a further culture period (72 h). This effect was sporadically observed in hCG-stimulated cultures (Figure 1 C, F). Interestingly, multinucleate cells ranged from 2-10 nuclei occurred in both hormonally stimulated cultures and unstimulated ones (Figure 1 B-F). The percentage of multinucleate cells is given in Table 1.

Immunocytochemical analysis

Using both Nomarski interference contrast and fine immunofluorescence analysis the positive immunostaining for LHR was localized at the cell plasma membrane level (Figure 1 G-L) of 24-and

48-hour Leydig cell cultures. The intensity of the staining was slightly stronger in hCG-stimulated cultures than in control ones (Figure 1 I-J, L). Irrespective of the number of Leydig cell nuclei, multinucleate cells were also positively stained for LHR (Figure 1 H, J, an insert in H). By 72 hour in culture Leydig cells displayed very weak staining for LHR or were immunonegative (*not shown*). There was no immunostaining for LHR when the primary antibody was replaced by normal goat serum (an insert in Figure 1 J). Typical distribution of microtubules (Figure 1 M-N) and microfilaments (Figure 1 O-P) was noticed irrespective of the number of nuclei as shown by immunofluorescence and DAPI, one of the most widely used nuclear counterstains (Figure 1 O-P). Sporadically, in the multinucleate cells stained for microtubules, nuclei appeared to be positively stained (Figure 1 N). In Leydig cells labeled with MitoTtracker Red CMXRos an abundance of mitochondria was observed in the cell cytoplasm in both mononuclear and multinucleate cells (Figure 1 P).

Table 1. The percentage of multinucleate Leydig cells in unstimulated and hCG-stimulated cultures. Data are presented as the mean ± SD (n = 4).

48h-Leydig cell cultures (n=4)	*Percentage of multinucleate Leydig cells*		
	2-4 nuclei	*5-7 nuclei*	*8-10 nuclei*
Unstimulated cells	13.75±0.96	6.75±0.96	3.25±0.50
hCG-stimulated cells	13.75±2.22	6.50±1.29	3.00±0.82

Table 2. Testosterone secretion by human Leydig cells *in vitro*. Data are presented as the mean ± SEM (n=4). The significance level was considered to be $p<0.05$. Means with the different letters (A, B) are statistically different (hCG-stimulated versus unstimulated cultures), whereas means with the different letters (a, b, c) within the row are statistically different between each of the time points of culture.

	Testosterone secretion by human Leydig cells in vitro [ng/10^6 cells/24h ± SEM]		
Cell cultures (n=4)	*24-hour*	*48-hour*	*72-hour*
Unstimulated cells	5.76±0.66^{A,a}	4.29±0.82^{A,b}	0.68±0.16^{A,c}
hCG-stimulated cells	9.83±1.18^{B,a}	6.85±1.03^{B,b}	0.84±0.18^{A,c}

Radioimmunological analysis

Radioimmunological analysis of testosterone levels revealed differences between testosterone secretion by hCG-stimulated and unstimulated Leydig cells. Human Leydig cells of unstimulated cultures secreted a lower amount of testosterone than Leydig cells of hCG-stimulated cultures ($p<0.05$). Moreover, the capability of hCG-stimulated and unstimulated Leydig cells *in vitro* to secrete testosterone diminished during the culture period. Differences were statistically significant at $p<0.05$ level (Table 2).

Discussion

Due to the rare availability of human material, the testis of rodents has long been utilized in studies on testicular structure and biosynthesis of androgens by Leydig cells (Preslock, 1980; Mather *et al.*, 1981; Zirkin *et al.*, 1997; Svechnikov *et al.*, 2001). There are several reports describing functional morphology of human Leydig cells *in vivo*, including studies on fetal, pre- and postpubertal testes (Nistal *et al.*, 1986a; Makabe *et al.*, 1995; Prince, 1990; Carreau, 1996; Chen *et al.*, 1996). Chemes *et al.* (1992) isolated human Leydig cell mesenchymal precursors to show the capacity of testosterone secretion and responsiveness to hCG of mesenchymal cells isolated from patients with androgen insensitivity syndrome. To date, however, there are only few data on morphology and function of human Leydig cells growing in a primary culture (Maillard *et al.*, 1994).

In our *in vitro* study, using both immunocytochemistry and fine immunofluorescence analysis, the positive staining for LHR was demonstrated at the cell plasma membrane level of the 24-and 48 hour-cell cultures. Very often, the staining in individual Leydig cells of the same culture varied in its intensity that could reflect a functional heterogeneity of Leydig cells *in vitro* as reported previously in Leydig cells of rodent testes (Kotula-Balak *et al.*, 2005; 2007). By 72 hours in culture Leydig cells displayed very weak staining for LHR or were immunonegative, suggesting that during culture the cells become hypofunctional. Perhaps the culture behavior could potentially reflect the *in vivo* situation, since it is well known that the presence of LHR is needed for a proper functioning of Leydig cells. Morphologically, hCG-stimulated cells appeared to be more regular in shape compared with the cells from unstimulated cultures. Interestingly, in Leydig cell population we observed multinucleate Leydig cells (23,20% and 22.80%) in both hCG-stimulat-

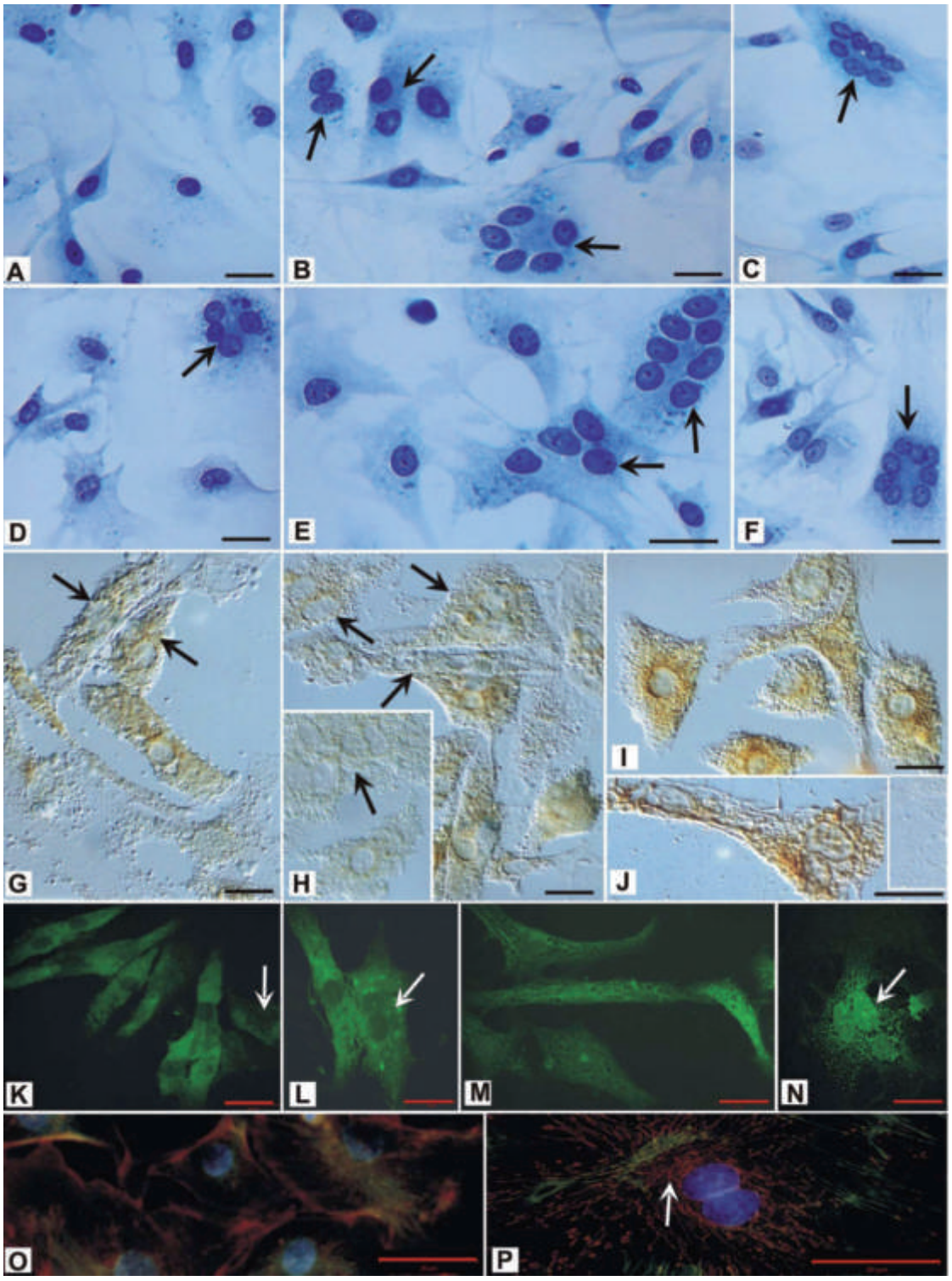

Figure 1. (A-P) Human Leydig cells *in vitro*. Bars = 20 µm. Morphology of M-GG stained Leydig cells in 24-hour-culture (A, D), 48-hour-culture (B, E), and 72-hour-culture (C, F). (A, B, C) unstimulated cultures. (D, E, F) hCG- stimulated cultures. Note the appearance of multinucleate cells in both culture conditions and during entire culture period (arrows). Immunostaining for LHR in 48-hour-old Leydig cells (G-L). Using Nomarski interference contrast (G-J) and fine immunofluorescence analysis the positive staining for LHR at the cell plasma membrane level was observed (K-L). Note a stronger intensity of the staining in hCG-stimulated cells (H-J, L) compared with that of unstimulated cells (G, an insert in H, K). Irrespective of the number of Leydig cell nuclei, multinucleate cells always positively stained for LHR (arrows). No staining for LHR observed when the primary antibody was omitted (an insert in J). Typical distribution of microtubules (M-N) and microfilaments (O-P) whatever the number of nuclei. Sporadically, after hCG stimulation, nuclei were positively stained for microtubules (arrow) (N). In Leydig cells labeled with MitoTtracker Red CMXRos an abundance of mitochondria was observed (arrow) (P).

ed and unstimulated cultures, respectively, what indicates that the formation of multinucleate cells is independent of hormonal stimulation *in vitro*. Multinucleate cells were found functionally active since they expressed LH receptors for the first two days of culture as did mononuclear Leydig cells. The existence of multinucleate Leydig cells in human testes has been a well known feature, however there are no data demonstrating the presence of multinucleate cells in culture. Schulze (1984) and Nistal *et al.* (1986b) described the presence of bi- or trinucleate Leydig cells as common in human testicular specimens. Using the PAP method the group of Nistal demonstrated functional activity of multinucleate Leydig cells *in vivo*. They reported a marked increase in the number of multinucleate Leydig cells with age, especially between the 4th to the 6th decade of life. In our study the testes were removed from elder than sixty-year-old patients, therefore it seems possible that the age of patients was a main cause of multinucleate cell appearance *in vitro*. Amat *et al.* (1986) provided ultrastructural evidence of mitosis in adult human cells suggesting that mitotic Leydig cells may contribute either to an increase in the number of Leydig cells or to the formation of multinucleate Leydig cells when karyokinesis without cytokinesis occurs. Multinuclearity of Leydig cells *in vitro* is difficult to discuss, however it is likely that it protects from effects of DNA damage. Multinucleate Leydig cells have also been observed in testicular disorders such as Klinefelter's syndrome and varicocele (Kotula-Balak *et al.*, 1994a; Aragona *et al.*, 1994).

Using a fluorescent mitochondrial marker we have shown an abundance of mitochondria in Leydig cells *in vitro* independently of the number of cell nuclei. Numerous mitochondria indicate functionally active cells as shown in mouse Leydig cells *in vitro* (Bilinska 1994; Kotula-Balak *et al.*, 2001). In earlier microscopic study at the ultrascructural level Chemes and co-workers (1992) reported that human Leydig cells possess an abundant smooth endoplasmic reticulum and steroid-type mitochondria. Of importance, Prince (1999) in his thorough study described tubulolamellar morphology of the mitochondrial cristae of human Leydig cells suggesting that the diversity of form of the cristae correlates structure and function in the process of steroidogenesis. The involvement of microtubules and microfilaments in steroidogenesis has been previously demonstrated in rodent Leydig cells *in vitro* (Bilinska, 1989; Bilinska *et al.*, 1997; 1999); therefore typical filament pattern in almost all human Leydig cells might reflect a normal functional state of the cultured cells.

Radioimmunological measurement of testosterone levels in human Leydig cells revealed a high testosterone secretion by the cells during the first 48 hour in culture. Moreover, it was significantly higher in the hCG-stimulated cultures compared with unstimulated ones, confirming several reports on the animal and human models (Simpson *et al.*, 1987; Chemes *et al.*, 1992; Maillard *et al.*, 1994; Rivarola *et al.*, 1995). After the next 24 hour in culture testosterone concentrations and the response to hCG stimulation of Leydig cells were very low, suggesting that deficits in individual Leydig cells may explain the age-related reductions in serum testosterone. This is also in agreement with earlier reports demonstrating that Leydig cells in primary culture rapidly undergo dedifferentiation (Purvis *et al.*, 1978; Mather *et al.*, 1981; Klinefelter and Ewing 1989; Maillard *et al.*, 1994). However, the enhancement of long-term testosterone secretion by human Leydig cells when co-cultured with human Sertoli cells has been observed by the group of Saez (Lejeune *et al.*, 1998). According to the authors, in humans, as in other species, Sertoli cells increase Leydig cell steroidogenic activity and a gonadotrophin FSH enhances the effect of Sertoli cells on Leydig cells. Additionally, a clear-cut response of cAMP to an acute hCG stimulation has been demonstrated by this group for both Leydig cells cultured alone and co-cultured with Sertoli cells. Based on reports to date on responsiveness to hCG of rat and human Leydig cells *in vitro* it may be concluded that the function of human Leydig cells is more similar to that of the rat than thought previously.

Finally, it can be added that culture of purified cell populations in defined media is often preferred to *in vivo* studies for the investigation of specialized cell functions as shown in the present study.

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