FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 42, No. 1, 2004
pp. 19-27

Possible involvement of microtubules and microfilaments of the epididymal epithelial cells in 17β-estradiol synthesis

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Abstract: The rat epididymal epithelial cells revealed features of steroidogenic cells and released 17β-estradiol (E2) into the culture medium. In steroidogenic cells, elements of the cytoskeleton due to their influence on organelle distribution are implicated in the regulation of steroidogenesis. In the present study, the morphology of cultured epididymal epithelial cells in light, scanning and transmission electron microscopes was evaluated. The organization of microtubules and microfilaments revealed by fluorescence microscopy, and the concentration of E2 in cultured medium were also studied. The epididymal epithelial cells were cultured in different conditions: in the medium with or without exogenous testosterone (T) and in the co-culture with Leydig cells as a source of androgens. The cells in co-culture located close to Leydig cells were rich in glycogen, PAS-positive substances and lipid droplets, in higher amount than the cells cultured with addition of exogenous testosterone. Stress fibers and microtubules of epididymal epithelial cells cultured with exogenous T and in co-culture with Leydig cells presented typical structure, and numerous granular protrusions appeared on the surface of the cells. Disorganization of microtubules and shortening of stress fibers as well as the smooth cell surface deprived of granular protrusions were observed in the epididymal epithelial cells cultured without T. Change of the cytoskeleton organization caused by the absence of androgen in culture medium resulted in an increased E2 secretion.

Key words: Epididymal epithelial cells - Culture - Cytoskeleton - Estradiol

Introduction

The morphology and the functional integrity of the epididymis are androgen-dependent. The hormones are produced mainly in testis and together with other hormones and factors are transported to the epididymis *via* rete testis fluid. The secretory activity of epididymal epithelial cells provides an environment for the post-testicular maturation of spermatozoa. The cells produce proteins, glycoproteins, glycolipids and phospholipids that are released in huge amounts into epididymal lumen mainly *via* apocrine secretion. Epididymal epithelial cells *in vitro* resume the functional activity but they need supplementation with androgens - testosterone (T) or 5α-dihydrotestosterone (DHT) [8, 13, 42]. Due to the presence of 5α-reductase, epididymal epithelial cells are able to convert testosterone to 5α-dihydrotestosterone [24, 34]. The morphology of cultured cells is depended on the presence of T or DHT in culture medium. When the epididymal epithelial cells are cultured in medium enriched with androgens, the cytoplasm accumulates lipid droplets, glycogen and PAS-positive substances.

The shape of cultured cells, cytoplasmic organization and surface structures are regulated by the elements of cytoskeleton. Both, microtubules and microfilaments influence the distribution of organelles and lipid droplets [9]. It is well documented, that in steroidogenic cells they are involved in the regulation of steroidogenesis [3, 9, 15]. The disarrangement of cytoskeletal elements, mainly microtubules and microfilaments, is observed in Leydig cells after stimulation with LH. Disruption of the cytoskeleton of cells, treated with disrupting drugs, leads to redistribution of mitochondria, lipid droplets and smooth endoplasmic reticulum and stimulation of steroid synthesis [27].

Cultured epididymal epithelial cells of rats have features of cells producing steroids. Their cytoplasm contains organelles and enzymes involved in the steroidogenic pathway. They are able to synthesize androgens that are converted by cytochrome P450 aroma-

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tase to 17β-estradiol [41, 42]. However, little is known on the behaviour of cytoskeleton in cultured epididymal epithelial cells during epididymal steroidogenesis both under influence of androgens and without supplementation with these hormones. Therefore, the aim of the present study was to estimate the morphology and the organization of microtubules and microfilaments in epididymal epithelial cells grown in media supplemented with exogenous testosterone (T), without testosterone, and in co-culture with Leydig cells as a source of T.

Materials and methods

Isolation of epididymal epithelial cells. Epididymides were obtained from adult Wistar rats, weighing 300-350 g each. The rats were maintained at controlled temperature (22-24˚C) and photoperiod of LD 12:12 h. The animals were anaesthetized with sodium pentobarbitone (100 mg/kg body weight).

The procedure for isolation and culture of epididymal epithelial cells was described previously [14] as a modified procedure of Kierszenbaum *et al.* [25]. Viability of the cells was detected by the trypan blue exclusion test. The isolated cells were transferred to plastic Petri culture dishes (Nunc Inc., Naperville, Il., USA) and cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, USA) supplemented with 5% inactivated fetal calf serum (FCS; Gibco BRL, Grand Island, USA) with or without 1 nmol/L testosterone (T; Sigma Chemical Co, St Louis MO, USA). They were cultured at 34˚C, in 5% CO_2 for 3 days, until they formed a monolayer. Thereafter, the medium was changed and the cells were cultured for the next two days. After that time, the cultures of epididymal epithelial cells were used for morphological evaluation.

Isolation of Leydig cells. Leydig cell suspension for co-culture was obtained by digestion of the testes with 0.25% trypsin for 15 minutes as previously described by Bilińska [4]. The tissue was serially sieved through 156 μm and 74 μm pore-size steel meshes and suspension of cells was centrifuged for 10 minutes at 180 × g. The supernatant was removed and the pelleted cells were resuspended in 1 mL of Dulbecco's medium supplemented with 5% FCS and antibiotics. Viability of the cells was detected by the trypan blue exclusion test.

Co-culture. Suspension of isolated Leydig cells was added to the epithelial cells of the caput and cauda epididymis cultured for 24 h in Dulbecco's modified Eagle's medium supplemented with 5% FCS without T. A co-culture of epididymal epithelial cells with Leydig cells were incubated at 34˚C, in 5% CO_2 for 48 h. Thereafter, the medium was changed and the cells were cultured for two days. After that time, co-cultures were used for morphological evaluation in the light microscope.

Morphological study. Both, the cultures of epididymal epithelial cells and co-cultures of the epididymal epithelial cells with Leydig cells were fixed in 60% isopropyl alcohol, and stained with Oil-Red-O for visualization of lipid droplets or with PAS method for visualization of neutral glycoproteins and glycogen [2].

To verify the culture homogeneity, the epithelial cells of caput and cauda epididymis were subjected to immunocytochemical staining for cytokeratin and desmin (DAKO, Glostrup, Denmark) [26, 39]. The immunolocalization of cytokeratin and desmin in the cultured cells was compared to immunolocalization in the epididymal sections.

To check the steroidogenic activity of cultured cells, the presence of Δ^5, 3β-hydroxysteroid dehydrogenase (3β-HSD) in the cytoplasm of cells in co-culture was detected using specific histochemical test. The incubation medium contained dehydroepiandrosterone (DHEA - Sigma Chemical Co, St. Louis MO, USA) as a substrate for 3β-HSD, NAD (Boehringer Mannheim Indianapolis, IN) as a co-factor and nitro blue tetrazolium (NBT - Sigma Chemical Co, St Louis MO, USA). To avoid a nonspecific reactivity, a control test was performed, using the medium without the substrate. The presence of formazan granules in the cytoplasm of the epididymal epithelial cells and the Leydig cells was a result of the reductive effect of NBT.

Visualization of microtubules and microfilaments. For immunofluorescence staining of microtubules and microfilaments, epididymal epithelial cells were cultured with or without T and co-cultured with Leydig cells. To study organization of microtubules after administration of an anti-microtubular agent, the epididymal epithelial cells were cultured with T for 3 days. Next, the cells were incubated in medium containing 250 μmol colchicine (Sigma Chem. Co. St. Louis, MO, USA) for 24 h. Colchicine was dissolved directly in culture medium.

All cultures were fixed with 3% formaldehyde and then permeabilized with Triton X-100 (Sigma Chem. Co. St. Louis, MO, USA). To stain the microtubules, the cultures were incubated first with mouse monoclonal antibody against tubulin (1:200; Sigma, USA) and then with fluorescein isothiocyanate-labelled goat anti-mouse IgG (Sigma, USA). To stain the microfilaments, the cultures were incubated with mouse monoclonal antibody against actin (1:80; Sigma, USA), followed by fluorescein isothiocyanate-labelled goat antimouse IgG (Sigma, USA). The control for specificity involved omission of the primary antibody and the cells were incubated only with 2% BSA. The specimens were evaluated using fluorescence microscope (Axioscop Zeiss, Germany) with filters for FITC (Filter set 09-487909-0000; Zeiss, Germany).

Scanning electron microscopy (SEM). The epididymal epithelial cells and co-cultures of epididymal epithelial cells with the Leydig cells were cultured in Petri dishes with 1×1 cm coverslips. After washing, the cultures were fixed with 0.25 mol/L glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, for 4 h at 4˚C, and postfixed in 0.04 mol/L OsO_4 for 12 h. Next, the specimens were dehydrated in a graded series of ethanol followed by acetone, dried in critical point drying apparatus using CO_2 as transition fluid (Polaron E3000), coated with gold-palladium (JEOL JEE) and observed in JEOL JSM 6100 scanning electron microscope.

Transmission electron microscopy (TEM). The caput and cauda epididymides were cut into 1 mm fragments, fixed in 0.25 mol/L glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, for 2 h at 4˚C, post-fixed in 0.04 mol/L OsO_4, dehydrated in ethyl alcohol (30-96%) and 100% acetone, and subsequently embedded in the Spurr low-viscosity embedding kit (Polysciences, Inc. Warrington, PA). Ultrathin sections were cut with Reichert OmU2 ultramicrotome, contrasted with uranyl acetate and lead citrate, and analysed in a JEM 1200 EX transmission electron microscope at 80 kV.

Hormone assays. The isolated epididymal epithelial cells were transferred to Dulbecco's medium supplemented with 5% FCS with or without T, and placed into 24-well cell culture dishes (Corning, NY, USA) always in the same number - 5×10^5 cells per well.

In case of co-culture, the epididymal epithelial cells were cultured in medium without T for one day. On the following day, the Leydig cells were added in amount of 2×10^5 cells per well. Media of epididymal epithelial cells cultured with and without T and co-cultures of epididymal epithelial cells with Leydig cells were changed on day 3 and cells were cultured for the next two days.

The media collected on day 3 and 5 of cell culture were centrifuged and frozen at -20˚C. They were subjected to measurements of 17β-estradiol (E2) level. Because the culture media were supplemented with 5% FCS, the concentration of E2 was also measured

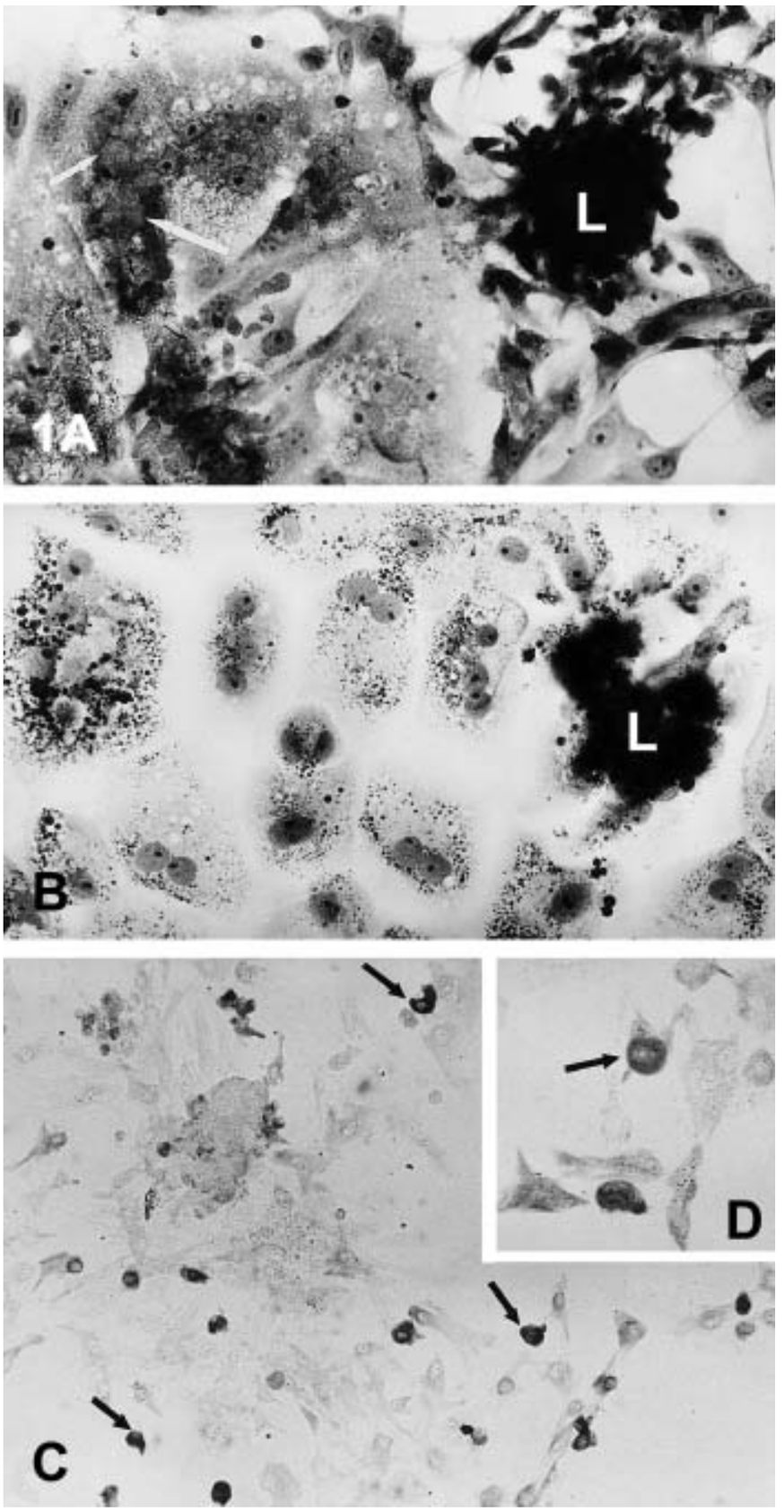


Fig. 1. Epididymal epithelial cells cultured in co-culture with Leydig cells (L) contain glycogen granules and PAS-positive substances (arrows) (Fig. 1A) and numerous lipid droplets (Fig. 1B). Positive reaction for 3β-HSD in the cytoplasm of epididymal epithelial cells and Leydig cells (arrows) (Fig. 1C,D). Fig. 1A - PAS, × 670; 1B - ORO, × 670; 1C,D - 3β-HSD, 1C × 160, 1D × 330.

in media before initiation of the cell culture. Levels of E2 were estimated with Enzyme Linked Fluorescent Assay (Bio Merieux, Marseilles, France) Vidas Estradiol II (E2II).

Statistical analysis. Results were expressed as means ± standard deviations (SD) and medians (M) with lower and upper quartiles (LQ-UQ). Non-parametric ANOVA Kruskal-Walis rank and Mann-Whitney U-test were used to check significance of differences. The value of $p<0.05$ was considered to indicate statistically significant differences. Calculations were done using the software package Statistica for Windows StatSoft, Inc., Tulsa, OK., USA.

Results

Morphology

After 3 days of culture, the segments of caput and cauda epididymides adhered to the bottom of plastic dishes. All the cells in culture were cytokeratin-positive. No desmin-positive cells (smooth muscle cells) were identified among the cultured cells [26, 39]. The epididymal epithelial cells cultured with exogenous testosterone (T) formed a confluent monolayer. The cells were rich in glycogen, PAS-positive substances and lipid droplets. The epididymal epithelial cells cultured without T did not form confluent culture. They had a stellate shape and were connected only by cytoplasmic processes. The cells were poor in glycogen and PAS-positive substances but contained lipid droplets [26, 39].

In the co-cultures, the Leydig cells formed clusters composed of different number of cells (Fig. 1A,B) and frequently they appeared as single cells among the epididymal epithelial cells (Fig. 1C,D). Epididymal epithelial cells located close to Leydig cells were more abundant in PAS-positive substances, glycogen granules (Fig. 1A) and lipid droplets (Fig. 1B) than the cells cultured with T only.

All cells revealed 3β-HSD activity. Formazan granules were observed both in the cytoplasm of Leydig cells and epididymal epithelial cells cultured with and without T. However, in Leydig cells the histochemical reaction appeared to be more intense (Fig. 1D).

Immunofluorescence staining of cytoskeleton

In the cytoplasm of epithelial cells of caput and cauda epididymis cultured with T and in co-culture with Leydig cells, typical arrangement of microtubules was observed. They spread from the region of nucleus forming a network (Fig. 2A,D). In the epithelial cells of caput and cauda epididymis cultured without T, disorganization of microtubules was observed. Free tubulin accumulated around the nucleus and the network pattern was partially destroyed (Fig. 2B). The incubation of the epididymal epithelial cells with colchicine resulted in complete depolymerization of microtubules and free tubulin formed granules (Fig. 2C). Localization of microfilaments revealed typical parallel stress fibers with actin condensation at the contact sites of the epididymal epithelial cells both cultured with T (Fig. 3A) and in co-culture with Leydig cells (Fig. 3C). Stress fibers of the epididymal epithelial cells cultured without T were shorter and diffuse actin staining was often seen (Fig. 3B).

Electron microscopy

Scanning electron microscopy revealed numerous granular protrusions on the surface of epididymal epithelial cells cultured with exogenous T (Fig. 4A), as well as on the cells in co-culture with Leydig cells (Fig. 4D). There were no protrusions on the surface of the epididymal epithelial cells cultured without T (Fig. 4C).

By transmission electron microscopy, stereocilia and protrusions filled with a lamellar material were observed in the apical part of the epididymal epithelial cells (Fig. 4B).

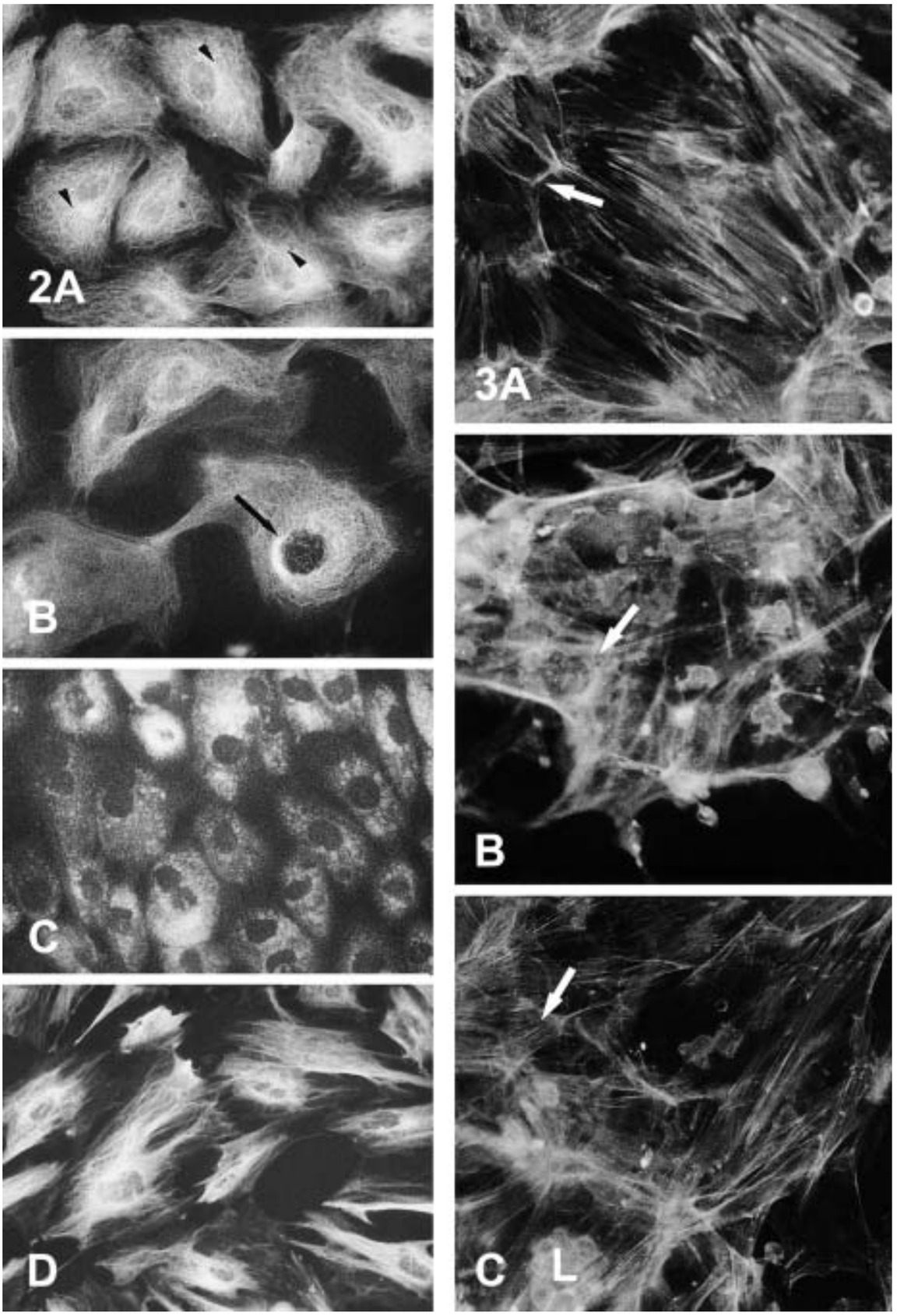
Hormone assay

The concentration of 17β-estradiol (E2) in the media of cultures where epithelial cells of the caput and cauda epididymides were cultured with addition of T was comparable to the concentration of E2 in the media of the cells cultured without T (Tab. 1). A high concentration of 17β-estradiol was detected in all culture media. The level of E2 in media supplemented with 5% FCS before initiation of culture was low – 21.36±2.9 pg/mL. We observed statistically significant differences in E2 concentration between media of epithelial cells of the cauda epididymides but not of caput cultured with and without T on day 3 of culture (Tab. 1).

There were no statistically significant differences between the concentrations of E2 in media of the caput and cauda epithelial cells cultured with or without T on day 5.

The concentration of E2 in media with co-cultures was not included to statistical comparison, because cultures of epididymal epithelial cells were carried out in different conditions.

Fig. 2. Typical arrangement of microtubules which spread from nuclear region (arrowheads) forming a network in cells cultured with testosterone (Fig. 2A) and in co-culture with Leydig cells (Fig. 2D). Disorganization of microtubules and free tubulin accumulation around the nuclei (arrow) in the epididymal epithelial cells cultured without androgens (Fig. 2B). Completely depolymerized microtubules in colchicine-treated cells (Fig. 2C). Fig. 2 A, B × 670; C,D × 400. **Fig. 3.** Stress fibers in the epididymal epithelial cells. Typical parallel stress fibers in the cytoplasm of cells cultured with testosterone (Fig. 3A) and in co-culture with Leydig cells (L) (Fig. 3C) with actin condensation at the site of contact of the cells (arrow). Shortening of stress fibers, diffuse actin staining and accumulation of actin (arrow) in the cytoplasm of cells cultured without androgens (Fig. 3B). Fig. 3 A, B, C × 670.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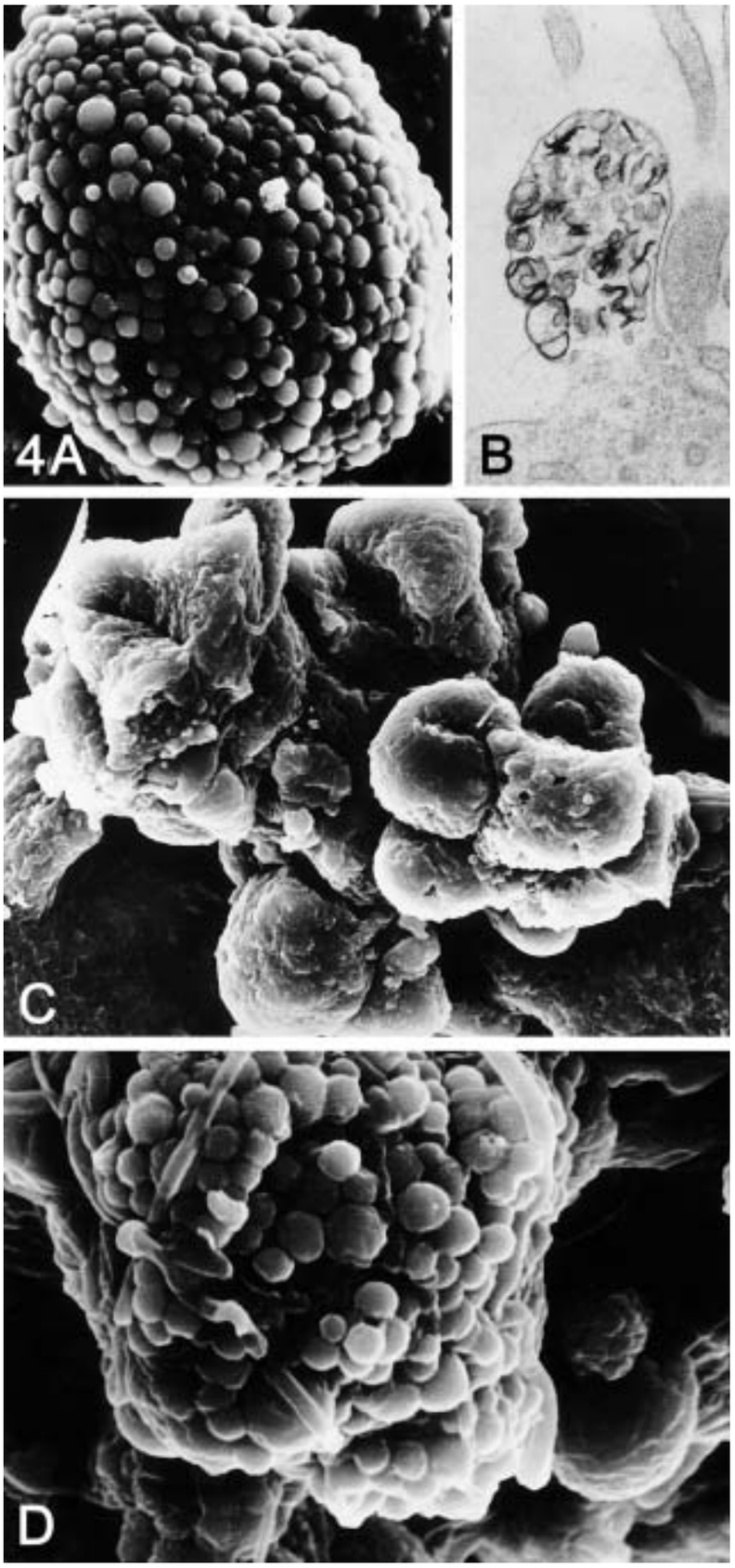

Fig. 4. Granular protrusions on the surface of epididymal epithelial cells cultured with addition of testosterone (Fig. 4A), and on the surface of the cells cultured in co-culture with Leydig cells (Fig. 4D). Smooth surface of the cells cultured without addition of T (Fig. 4C). Stereocilia and a protrusion containing lamellar material on the surface of cell of caput epididymis epithelium (4B). Fig. 4. SEM, × 4620 (A); × 4620 (C), × 4000 (D); TEM, × 30000 (B).

Table 1. Concentrations of 17β-estradiol (E2) in media of epididymal epithelial cells cultured with or without T, and in co-culture with Leydig cells

Culture	Culture with testosterone (T)		Culture without testosterone (T)		Co-culture	
	Mean ± SD	M (LQ-UQ)	Mean ± SD	M (LQ-UQ)	Mean ± SD	M (LQ-UQ)
Epididymal caput E2 (pg/mL) day 3	658.9 ± 137.4	720.1 (512.5 - 758.6)	819.0 ± 82.6	773.3 (767.4 - 847.4)	206.2 ± 5.5	208.7 (206 - 208.8)
Epididymal cauda E2 (pg/mL) day 3	361.4 ± 86.3	337.8 (333.1 - 337.8)	569.5 ± 191.9*	611.4 (403.7-707.0)*	245.3 ± 27.6	237.4 (237.4 - 252.1)
Epididymal caput E2 (pg/mL) day 5	564.5 ± 30.4	561.8 (547.6 - 576.4	639.1 ± 75.4	635.1 (574.8 - 708.5)	143.7 ± 9.2	139.5 (136.9 - 149.5)
Epididymal cauda E2 (pg/mL) day 5	282.1 ± 41.3	274.5 (251.1 - 282.4)	426.5 ± 142.3	447.8 (312.5 - 510.2)	216.2 ± 38.9	223.2 (185.3 - 247.4)

Means ± SD and medians (lower-upper quartiles) [M (LQ-UQ)]; n = 5 ; *$p<0.05$.
The results of E2 concentration in media of co-cultures were not compared to E2 concentration in media of the epididymal cells cultured without or with T, since both epididymal epithelial cells and Leydig cells were able to produce 17β-estradiol in co-culture.

Discussion

The epididymis is an hormone-dependent organ. The morphology and secretory activity of epididymal epithelial cells is maintained mainly by androgens acting through androgen receptors. However, the human and animal epididymal epithelial cells contain also both α and β estrogen receptors [10, 11, 12, 29, 36]. It is postulated that the function of the epididymis is regulated not only by androgens but additionally by estrogens [16, 21, 22, 43]. At present it is known that estrogens are produced in testis [10, 11, 12, 18], epididymis [42], and in spermatozoa [23, 33].

The experimental deprivation of androgens reduced the height of epithelium and induced changes of morphology and secretory activity in the epithelial cells of the animal epididymis [19]. Therefore, to maintain the typical morphology and function, the epididymal epithelial cells should be cultured in a medium supplemented with androgens [13, 42].

We demonstrated in our former and present studies, that isolation method yielded homogenous population of the epididymal epithelial cells [26, 39]. When the cells were cultured with addition of exogenous T or DHT, they formed a confluent monolayer and resumed their function [26, 39, 41, 42]. They were rich in glycogen, PAS-positive substances and lipid droplets. The cells cultured without exogenous androgen did not form a confluent monolayer and were poor in glycogen and PAS-positive substances, but they contained lipid droplets. They synthesized androgens but in amount unable to maintain a typical morphology [41, 42]. In the present experiments, the epididymal epithelial cells were cultured with Leydig cells as a source of androgens. The Leydig cells expressed 3β-HSD indicating their steroidogenic function. The epididymal epithelial cells in co-culture with Leydig cells formed also a confluent monolayer and accumulated lipid droplets, glycogen and PAS-positive substances. All of them were produced in much higher amount then in the cells cultured with T only. It was an expected effect, because co-culture with Leydig cells provided the most similar conditions to those *in vivo*. In addition, the Leydig cells could be the source of other substances which influence the epididymal epithelial cells [35].

The organization of cytoskeletal elements in cells cultured in a medium supplemented with exogenous T or androgens released by Leydig cells was typical. The cytoskeleton was changed when the cells were cultured without T. The lack of androgen in the medium caused spatial disorganization of microtubules and their partial depolymerization. However, the picture of microtubules in the cells differed from that in colchicine-treated cells, where the microtubules were completely depolymerized and free tubulin granules were present. The observation indicated, that the mechanism of microtubule disorganization in colchicine-treated cells was different from that caused by the lack of testosterone in culture medium. Moreover, shortening of microfilaments and accumulation of actin in epididymal epithelial cells cultured without addition of exogenous testosterone were observed. The changes in organization of cytoskeleton were associated with changes of the cell surface. The surface of cells cultured with T and in the co-culture showed the presence of granular protrusions, as observed in epididymal epithelial cells in transmission electron microscopy both *in vivo* and *in vitro* [40]. The surface of epididymal epithelial cells cultured without androgen was smooth, and devoid of granular protrusions. The granular protrusions on the surface of epididymal epithelial cells reflected the apocrine secretion, and the process was androgen-dependent. It is well documented that the epididymal epithelial cells reveal apocrine secretion [7, 20, 31, 32, 40].

The ultrastructure of rat epididymal epithelial cells *in vitro* revealed features of steroidogenic cells [41]. Like

in other animal and human epididymides [37], the epithelial cells have active enzymes of the steroidogenic pathway [41]. Cultured both with or without T and DHT, they were able to synthesize androgens and converted them to 17β-estradiol, due to the presence of cytochrome P450 aromatase [41, 42]. However, the quantity of synthesized androgens was small, they were released sometimes in trace concentrations and were unable to maintain the morphology of the epididymal epithelial cells in culture [42].

The changes in the distribution of microtubules and microfilaments in epididymal epithelial cells cultured in medium without T promoted synthesis of E2. The concentration of E2 in media before initiation of cell culture was 21.36±2.9 pg/mL. The concentration of E2 in media of epithelial cells cultured without T was very high. The observed effect was similar to the regulation of steroidogenesis in Leydig cells after LH stimulation [28, 30] or after treatment with disrupting drugs, *e.g.* colchicine [30]. LH stimulation of Leydig cells is known to enhance steroidogenesis [28, 30], which is preceded by the disarrangement of cytoskeletal elements, mainly microtubules and microfilaments [4, 5, 6, 30].

It is likely that as in Leydig cells, the disarrangement of the cytoskeletal elements in the epididymal epithelial cells alters distribution of mitochondria, lipid droplets and smooth endoplasmic reticulum and stimulates steroidogenesis. This is difficult to explain while the epididymal epithelial cells cultured without T revealed such high levels of E2. We suggested that epididymal steroidogenesis is directed towards the synthesis of 17β-estradiol [42]. It might be expected that the process of estradiol synthesis, could be independent of exogenous androgens, thus protecting the cells against the absence of this hormone.

As mentioned above, the epididymal epithelial cells in co-culture stayed in conditions similar to those *in vivo*. The Leydig cells produce not only androgens but also other hormones, *e.g.* oxytocin [1]. The epididymal epithelial cells can express receptors for oxytocin, as demonstrated in ram epididymis [38]. Oxytocin inhibited testosterone synthesis in cultured Leydig cells [17]. Similar effect can take place in epididymal epithelial cells in co-culture with Leydig cells and result in lower concentration of E2 in medium. On the other hand, the epididymal epithelial cells in co-culture with Leydig cells may have been unable to convert Leydig cell androgens into E2, because the epididymal epithelial cells express a different form of cytochrome P450 aromatase than Leydig cells [42].

The present studies extend the knowledge of epididymal steroidogenesis and suggest participation of cytoskeletal elements in this process.

Acknowledgements: The research was supported by the State Committee for Scientific Research (KBN) as a Solicited Project PBZ-KBN-084/P06/2002 from 2003 to 2005.

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Accepted October 2, 2003