Binding of lipid droplets and mitochondria to intermediate filaments in rat Leydig cells

G. Almahbobi, L. J. Williams, X-G. Han and P. F. Hall

Department of Endocrinology, Prince of Wales Hospital, Randwick 2031, NSW, Australia

We have examined the distribution of lipid droplets and mitochondria in relation to the cytoskeletons of Leydig cells in primary culture by using light and electron microscopy on living, intact and detergent-extracted cells. After mild extraction with Triton X-100 lipid droplets and mitochondria retained their original distribution within the cell. Double immunofluorescent microscopy showed that both structures co-localise with intermediate filaments. Transmission electron microscopy of intact (unextracted) and mildly extracted Leydig cells showed that intermediate filaments are closely associated with mitochondria and lipid droplets. By examination of stereo pairs, intermediate filaments were shown to establish direct contact with mitochondria and lipid droplets. The association of droplets and mitochondria with intermediate filaments suggests possible mechanisms by which the transport of cholesterol takes place from droplets to mitochondria where this substrate enters the steroidogenic pathway.

Introduction

It is generally agreed that the slow step in steroid synthesis involves the transport of cholesterol from cytoplasmic stores to the inner mitochondrial membrane where steroidogenesis begins with the conversion of cholesterol to pregnenolone (side-chain cleavage) (Hall, 1987). The cytoplasmic stores of cholesterol include lipid droplets that are characteristic of steroidogenic cells (Silberzahn *et al.*, 1985; Almahbobi *et al.*, 1985, 1988; Almahbobi and Hall, 1990). The process of cholesterol transport involves the cytoskeleton and, in particular, actin (Hall *et al.*, 1979; Osawa *et al.*, 1984). The mechanism by which the cytoskeleton promotes this transport is not clear. We have reported that lipid droplets (Almahbobi and Hall, 1990; Almahbobi *et al.*, 1992a) and mitochondria (Almahbobi *et al.*, 1992b) are attached to intermediate filaments in adrenal cells. Since attachment of these structures to intermediate filaments may be important in the regulation of cholesterol transport from droplets to mitochondria, it appeared important to determine whether a similar association is found in other steroidogenic cells. We show here that droplets and mitochondria are bound to intermediate filaments in rat Leydig cells in primary culture.

Materials and Methods

Materials

Materials were purchased as follows: Ham's F12, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and horse serum from Flow Laboratories Inc., Irvine; Percoll from Pharmacia LKB, Biotechnology AB, Uppsala; Nile red from

Eastman Kodak Company, Rochester, NY; collagenase type IA, deoxyribonuclease I (DNAase I), BSA, rhodamine 123, immunoglobulin Gs (IgGs) of uninjected animals, goat anti-vimentin and rabbit anti-goat-fluorescein isothiocyanate (FITC) antibodies from Sigma Corporation (St Louis, MO). Mouse anti-vimentin, sheep anti-mouse-FITC and donkey anti-rabbit-Texas red antibodies were purchased from Amersham International (Buckinghamshire). Goat anti-mouse IgG + IgM antibody conjugated to 5 nm gold particles was obtained from Janssen (Life Sciences Products, Beerse). Rabbit anti-cytochrome oxidase antibody was a gift from E. Racker, Cornell University, Ithaca, NY. The specificity of this antibody has been described elsewhere (Eytan *et al.*, 1975). All secondary antibodies were affinity purified as stated by suppliers.

Cell isolation and cultures

Sprague-Dawley rats, aged 30 days, were killed by cervical dislocation. The testes were removed aseptically and immediately transferred into ice cold medium (Ham's F12/DMEM, 1:1). All other procedures were carried out under sterile conditions. Leydig cells were prepared by a published method (Browning *et al.*, 1981) with modification. After decapsulation, testicular tissue was incubated for 30 min at 37°C with gentle shaking in the same medium containing BSA (1 mg ml⁻¹), DNAase I (10 µg ml⁻¹) and collagenase type I (0.5 mg ml⁻¹). Leydig cells were isolated by means of centrifugation (800 g for 20 min) through a continuous Percoll gradient. After washing twice, the cells were plated in plastic culture dishes, glass slide chambers or on coverslips. The cultures were maintained in medium supplemented with 12.5% horse serum, 2.5% FCS and gentamycin A (32 mg ml⁻¹) in a humidified atmosphere of 95% air: 5% CO₂ for 2–3 days to obtain cells that were well spread, firmly attached and confluent. The purity of our preparations

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of Leydig cells was greater than 95% as determined by histo-enzymological staining for 3β -hydroxysteroid dehydrogenase. Production of testosterone with ($46 \text{ pmol per } 10^6 \text{ cells h}^{-1}$, $n = 3$) and without ($24 \text{ pmol per } 10^6 \text{ cells h}^{-1}$, $n = 3$) dibutyryl cAMP was measured by radioimmunoassay after 24 h of culture, respectively.

Cell extraction

Cells grown in slide chambers, on coverslips, on gold grids and in dishes were subjected to extraction with buffered Triton X-100 as described by Almahbobi and Hall (1990) and Fey *et al.* (1984). The concentration of Triton and the duration of extraction were varied. Extraction of Leydig cells with 0.5% Triton for 5 min gave the best recovery of mitochondria and excellent immunofluorescent staining of all three cellular structures studied (Almahbobi *et al.*, 1992b). In some experiments intermediate filaments were prepared as described by Almahbobi and Hall (1990) and Fey *et al.* (1984). Intermediate filaments were prepared by extracting cells with 1% Triton X-100 for 10 min followed by 1% Triton X-100 containing ammonium sulfate (Fey *et al.*, 1984).

Light microscopy

Lipid droplets and mitochondria of living cells grown in slide chambers were stained with Nile red (Greenspan *et al.*, 1985; Almahbobi and Hall, 1990) and rhodamine 123 (Johnson *et al.*, 1980; Almahbobi *et al.*, 1992b), respectively. Nile red was used at a concentration of 100 ng ml^{-1} for 10 min and rhodamine 123 at $10 \text{ } \mu\text{g ml}^{-1}$ for 30 min at 37°C . Immunofluorescent staining was performed on extracted cells grown in slide chambers. Cells were fixed in 3.6% formaldehyde v:v in phosphate buffer saline (PBS, 0.01 mol l^{-1} , pH 7.2) for 30 min. For double staining of lipid droplets and vimentin-intermediate filaments, mouse anti-vimentin monoclonal antibody (1:5 for 60 min) and sheep anti-mouse antibody conjugated to FITC (1:40 for 60 min) were used. After extensive washes with PBS, cells were incubated in Nile red as described above. Other details of these procedures have been described elsewhere (Almahbobi and Hall, 1990).

Double indirect immunofluorescence to stain mitochondria and vimentin-intermediate filaments was performed using a modified procedure (Almahbobi *et al.*, 1992b; Almahbobi and Hall, 1993) in which four antibodies (two primary and two secondary) raised in three species were used in the following order: rabbit anti-cytochrome oxidase (1:30 for 60 min), wash with PBS, donkey anti-rabbit-Texas red (1:40 for 40 min), wash with PBS, goat anti-vimentin (1:20 for 60 min), wash with PBS, rabbit anti-goat-FITC (1:20 for 40 min), extensive wash with PBS. After this procedure, mitochondria were stained with both fluorochromes, while intermediate filaments were stained only by FITC. Both intermediate filaments and mitochondria are displayed with one light filter (blue); this makes it possible to demonstrate co-localisation of these two structures. However, one of these structures (mitochondria), but not the filaments, was identified by using a second filter (green). A number of control conditions were used for each staining i.e. no primary antibody, no secondary antibody, IgG from uninjected animals and antibody preabsorbed with antigen. The above procedures were all performed at room temperature unless otherwise stated.

Electron microscopy

Intact and extracted cells grown on coverslips were prepared for conventional electron microscopy as monolayers. Cells were fixed in 1/2 Karnovsky fixative and then processed as described by Almahbobi *et al.* (1988, 1992a, b). Thin sections were examined with a Hitachi 7000 transmission electron microscope. Stereopair micrographs were made by means of tilting.

Immunoelectron microscopy

Immunoelectron microscopy was performed on whole mount preparations of intermediate filaments from cells grown on Formvar-coated gold grids. Mouse anti-vimentin antibody was used at a dilution of 1:5 for 60 min followed by goat anti-mouse antibody conjugated to 5 nm gold particles (1:25) for 30 min. All other procedures were as described by Almahbobi and Hall (1990). In some experiments post-fixation with osmium tetroxide was omitted. The freeze dried preparations were examined with a Hitachi 7000 transmission electron microscope.

Results

Light microscopy

Figure 1 shows living cultured rat Leydig cells (a and b) and those extracted with Triton X-100 (c and d). Lipid droplets are revealed by a specific lipid stain, namely Nile red (Greenspan *et al.*, 1985) and mitochondria are stained by rhodamine 123 which has been widely used to specifically identify mitochondria (Johnson *et al.*, 1980). Lipid droplets appear as spherical structures and in some cases they are arranged in linear array extending from the nucleus (Fig. 1a, arrow) which results from association with filamentous structures. By contrast, mitochondria are oval or elongated and appear to be concentrated in the perinuclear region of the cell (Fig. 1b). When the cells are extracted with Triton X-100 (0.5%, 5 min), both organelles remain with the resulting cytoskeleton (Fig. 1c, d). The appearance and distributions of both structures are similar to those seen in the living cells indicating that the extraction procedure has not produced redistribution of either droplets or mitochondria, although some mitochondria have been broken into smaller segments by treatment with Triton X-100 (Fig. 1d). The extraction procedure has cleared the cells of cytoplasm and other structures so that the cytoskeletons show higher resolution of the stained structures (Fig. 1c, d).

Double indirect immunofluorescence was used to reveal lipid droplets and vimentin-intermediate filaments or mitochondria and vimentin-intermediate filaments (Fig. 2a, b and c, d, respectively). The relationship between droplets and vimentin-intermediate filaments is shown (Fig. 2a). Droplets are seen only in proximity to the filaments and careful examination shows that individual droplets are seen along the course of a given filament. With the blue filter, the droplets are clearly identified by shape and can be distinguished from the accompanying filaments (Fig. 2a). Only the lipid droplets can be seen when the sample is viewed with the green filter (Fig. 2b). Mitochondria are seen to be distributed along the course of intermediate filaments suggesting an association between these structures (Fig. 2c, d). These associations are seen at high magnification (Fig. 3)

Fig. 1. Fluorescence and immunofluorescence staining of living and extracted Leydig cells. (a) and (b): living cells; (c) and (d): extracted cells (0.5% Triton, 5 min). In (a) and (c) lipid droplets are stained with Nile red; in (b) mitochondria are stained with rhodamine 123 and in (d) mitochondria labelled with anti-cytochrome oxidase and second antibody conjugated to FITC. Arrow indicates lipid droplets arranged in linear array; n: nucleus. Bar = 5 μ m.

which shows fields from cells treated as for Fig. 2a and c. It is clear that droplets are distributed along the course of filaments and that mitochondria are superimposed upon filaments so that the two structures are not distinctly separated (arrows). The co-localization of droplets and mitochondria with intermediate filaments is sufficiently strong to withstand extraction with detergent and extensive washing. It should be noted that lipid droplets and mitochondria are seen only in those regions of the cells in which vimentin-intermediate filaments are present. This excludes the possibility that co-localization between these organelles and intermediate filament is due simply to non-specific crowding of intracellular contents. Co-localization between droplets and mitochondria on the one hand and micro-filaments or microtubules on the other hand was never observed.

Electron microscopy

Intact cells. Transmission electron microscopy of a single rat Leydig cell is shown (Fig. 4). The most prominent organelles are lipid droplets and mitochondria. In addition, the usual organelles are present. The appearance and distribution of lipid droplets and mitochondria throughout the cell are consistent with the findings with light microscopy described above. The upper inset shows numerous intermediate filaments that make direct contact with lipid droplets. The lower inset shows similar contact between intermediate filaments and mitochondria. The diameters of these filaments were found to be 10.0 ± 2 nm (mean and range, $n = 100$) which is the accepted range for intermediate filaments. This cell is representative of many Leydig cells subjected to thin section without any extraction procedure.

Extracted cells. When the Leydig cells were extracted with Triton X-100 under mild conditions (0.5% v:v for 5 min) cytosol was removed from the cells (Fig. 5a). Lipid droplets, mitochondria and endoplasmic reticulum are seen. However, the extraction procedure damaged the outer mitochondrial membrane so that these organelles did not retain their usual morphology. The inner structures of these organelles have rounded up into spherical structures; the endoplasmic reticulum is fragmented and dilated, but the extraction procedure has not changed the appearance of lipid droplets.

More extensive extraction (1% Triton for 10 min, then 1% Triton plus ammonium sulfate, for 10 min) of the Leydig cells results in a preparation of intermediate filaments from which the remaining cytoskeletal elements have been removed. Under these conditions, lipid droplets remain associated with the filaments and all other cellular structures, including mitochondria, have been removed (Fig. 5b). The nuclear skeleton can be seen in such preparations (Fig. 5b). It should be noted that the various extraction procedures from mild to severe do not release increasing proportions of all internal structures but are selective in what is released. For example, plasma membrane can be largely removed by very mild extraction. More severe extraction leaves mostly mitochondria, droplets and filaments. Finally, with severe extraction, only droplets and intermediate filaments remain. Some of these stages are illustrated here (Figs 5a, b, 6 and 7). Clearly mitochondria and lipid droplets are not non-specifically trapped in a meshwork of intermediate filaments.

Contact sites. Stereo pairs were prepared from severely extracted Leydig cells to demonstrate direct contact between filaments and lipid droplets (Fig. 6a, b). With the stereoscope, it is possible to see that some filaments end in contact with the droplet and hence both structures are located in the same plane within the thickness of the section (arrows). Similar studies were performed to show direct contact between filaments and mitochondria in mildly extracted cells (Fig. 7a, b, arrows).

Immunoelectron microscopy

The distribution of gold particles (5 nm) in non-osmicated preparations treated with anti-vimentin is shown (Fig. 8a). Gold particles are seen along the course of 10 ± 2 nm filaments (mean and range, $n = 100$). Non-osmicated preparations display a high contrast between the gold particles and the

Fig. 2. Double staining of extracted (0.5% Triton, 5 min) Leydig cells. (a) Lipid droplets and vimentin-intermediate filaments; (b) lipid droplets of the same cell as in (a); (c) mitochondria and vimentin intermediate filaments; (d) mitochondria of the same cell as in (c). (a) and (c): blue filter; (b) and (d): green filter. Arrows indicate lipid droplets (a, b) or mitochondria (c, d). n: nucleus. Bar = 2 μ m.

Fig. 3. High magnification of double staining of extracted (0.5% Triton, 5 min) Leydig cells. (a) Lipid droplets and vimentin-intermediate filaments; (b) mitochondria and vimentin-intermediate filaments. Arrows indicate (a) lipid droplets or (b) mitochondria. Bar = 1.4 μ m.

underlying filaments. From our experience, intermediate filaments become very darkly stained after treatment with osmium tetroxide. This largely reduces the contrast and, therefore,

obscures the visibility of the gold particles. When no first antibody (Fig. 8b) or normal mouse serum were used, no gold particles were seen. In considering the diameters of intermediate

Fig. 4. Electron microscopy of intact Leydig cells. Low magnification of a Leydig cell in primary culture is shown. Upper inset: high magnification of the area limited by a rectangle in the main micrograph showing the direct contact between intermediate filaments and lipid droplets. Lower inset: direct contact between intermediate filaments and mitochondria. Arrows: intermediate filaments; *: endoplasmic reticulum; l: lipid droplet; m: mitochondria; n: nucleus. Bar = 0.3 μ m (main), 0.1 μ m (insets).

filaments, it is necessary to consider at least three factors that may influence the apparent diameters of these structures. First, intermediate filaments may associate with each other in bundles. Second, the process of shadowing leaves a layer of the shadowing material along the course of some filaments. Third, the preparation of intermediate filaments may result in the deposition of a proteinaceous material of unknown origin along the filaments. Careful examination will reveal the true intermediate filament as a dark core within the coated structure especially in osmicated preparation (Fig. 8b). These factors were all taken into consideration in estimating the true diameter of the structures in our preparations as 10 ± 2 nm. In this connection it should be noted that the gold particles indicating the presence of anti-

vimentin (and hence vimentin itself) are confined to the central region of the filaments.

Discussion

The present studies are based upon primary cultures of rat Leydig cells, the ultrastructure of which has not been reported. It is known that rat Leydig cells in culture retain their normal steroidogenic activity for up to two (Rommerts *et al.*, 1982) or three (Mather *et al.*, 1981) days and then production of androgens starts to decline. Our findings confirm this conclusion as judged by the production of testosterone with and without

Fig. 5. Electron microscopy of extracted cells. (a) Mild extraction (0.5% Triton, 5 min), and (b) intermediate filaments preparation (1% Triton, 10 min then 1% Triton plus ammonium sulfate, 10 min). Arrow: intermediate filaments; arrowhead: residual cell membrane; *: dilated endoplasmic reticulum; l: lipid droplet; m: mitochondria; n: nucleus. Bar = 0.25 μ m.

Fig. 6. Stereo pair electron microscopy of severely extracted (1% Triton, 10 min) Leydig cells. Arrows indicate the intermediate filaments that are in direct contact with a lipid droplet photographed at (a) -12 and (b) $+12$ degrees of tilting. Bar = 0.08 μ m.

Fig. 7. Stereo pair electron microscopy of Leydig cells after mild extraction (0.5% Triton, 5 min). Arrows indicate the contact points between intermediate filaments and a mitochondrion photographed at (a) -12 and (b) $+12$ degrees of tilting. Bar = $0.08 \mu\text{m}$.

dibutyryl cAMP. The ultrastructure of the cells shown after 3 days in culture reveals that the cells and their steroidogenic organelles are very similar to those of cultured adrenal cells (Almahbobi *et al.*, 1992a, b). The structures described here as vimentin-intermediate filaments have been identified by three criteria. First, in extracted cells, filamentous structures reacting with anti-vimentin were seen. Second, structures of similar appearance and distribution were of the diameter characteristic of intermediate filaments i.e. $10 \pm 2 \text{ nm}$ (means and range, $n = 100$). Third, these structures were identified by immunoelectron microscopy. Moreover, the intermediate filaments were prepared by a method known to yield only these structures in other cells (Fey *et al.*, 1984).

In these studies we have investigated the association between two intracellular structures necessary for steroid synthesis, namely lipid droplets and mitochondria with vimentin-

intermediate filaments. Cholesterol is stored as ester in lipid droplets and must be cleaved to give free cholesterol which is transported to the inner mitochondrial membrane where steroid synthesis begins. This process of transport is of particular importance because it involves the rate-limiting step in steroid synthesis – one that is specifically stimulated by LH and ACTH (Hall *et al.*, 1979; Osawa *et al.*, 1984; Hall, 1987). The process is poorly understood but is known to involve the cytoskeleton (Hall *et al.*, 1979; Osawa *et al.*, 1984; Hall, 1987). It is, therefore, of considerable interest to learn that the droplets are tightly attached to intermediate filaments. In attempting to explain the resistance of the lipid content of the droplets to detergents and other agents used in electron microscopy, we found that in adrenal cells the lipid is protected by a capsule containing vimentin (Almahbobi and Hall, 1990; Almahbobi *et al.*, 1992a). It seems likely that the same protection of droplets occurs in Leydig cells

Fig. 8. Immunoelectron microscopy of intermediate filaments preparation (1% Triton, 10 min then 1% Triton plus ammonium sulfate, 10 min) of cultured Leydig cells. The filaments were prepared as whole mounts and were stained with (a) anti-vimentin antibody followed by secondary antibody conjugated to gold particles (5 nm). Although filaments appear thicker than the normal diameter in such a preparation, gold particles are seen along the central part (10 ± 2 nm) of the filaments. This preparation was not post-fixed with osmium tetroxide. (b) Control preparation with no first antibody. This preparation was treated with osmium tetroxide. l: lipid droplet. Bar = 0.05 μ m.

which clearly resist the same treatments. As in adrenal cells, the droplets are 0.2 to 3 μ m in diameter and are usually roughly spherical (Almahbobi and Hall, 1990).

Attachment of lipid droplets to intermediate filaments was demonstrated in Leydig cells by several methods. First, in immunofluorescence, lipid droplets are seen along the course of filaments and not in regions of the cell where there are no filaments. Second, electron microscopy of intact cells shows filaments apparently touching lipid droplets. This association is more obvious when cells are cleared by extraction with Triton X-100. Finally, direct contact between these structures can be seen in stereo pairs with tilting. This approach offers the most convincing evidence of direct contact because it provides a three-dimensional image of the structures in question. In non-steroidogenic cells, tight association between lipid droplets and intermediate filaments has been reported in breast adipocytes (Franke *et al.*, 1987).

Attachment of mitochondria to intermediate filaments is strongly suggested by the appearance of the mitochondria along the course of the filaments. Mitochondria are not seen at a distance from filaments on immunofluorescence. The apposition of elongated mitochondria with filaments results in the appearance of a composite structure consisting of filaments and mitochondria in close association. Transmission electron microscopy also shows close association between these structures and, once again, direct contact is established by means of stereo pairs. Close association between intermediate filaments and mitochondria has been reported in Leydig cells (David-Ferreira and

David-Ferreira, 1980), in hepatocytes (Fiskum *et al.*, 1980), in HeLa cells and aortic smooth muscle cells (Lin *et al.*, 1990) and in fibroblasts (Mose-Larsen *et al.*, 1982).

Extraction of Leydig cells with Triton does not disturb the attachment of droplets to intermediate filaments but destroys the outer mitochondrial membrane and, hence, releases mitochondria from the filaments. However, indirect immunofluorescence with anti-cytochrome oxidase following such extraction reveals some traces of fluorescent staining, probably associated with intermediate filaments, suggesting that mitochondria may be tightly attached to intermediate filaments (not shown). This finding was also made with adrenal cells (Almahbobi *et al.*, 1992b). This means that the attachment of mitochondria to filaments is not less tenacious than that between droplets and filaments but rather that the mitochondrial membrane is susceptible to the action of Triton.

Since the first step in the conversion of cholesterol to steroid hormones involves transport of that substrate to mitochondria and since the cytoskeleton is required for this process, the attachment of both the source of cholesterol (droplets) and the site of its conversion to pregnenolone (mitochondria) to the same filamentous component of the cytoskeleton is likely to be of functional importance. Earlier reports in the adrenal cortex show droplets in close physical contact with mitochondria (Merry, 1975). Moreover, evidence for an association between vimentin-intermediate filaments and smooth endoplasmic reticulum has been reported in Leydig cells of rat testicular tissue (Russell *et al.*, 1987). The fact that the three main steroidogenic organelles (lipid droplets, mitochondria and endoplasmic

reticulum) are associated with vimentin-intermediate filaments highlights the significance of these findings. Indeed, as Steinert and Liem (1990) point out, intermediate filaments far from being inert, supporting structures are extremely dynamic, and participate in numerous cellular activities. Vimentin-intermediate filaments play a functional role in the intracellular transport of lipoprotein-derived cholesterol in human SW-13 adrenal tumour cells (Sarria *et al.*, 1992).

Studies from this laboratory (Hall *et al.*, 1979; Osawa *et al.*, 1984; Hall, 1987) showed that actin microfilaments are required for the increase in cholesterol transport produced by LH and ACTH. A combination of some contractile event involving actin and the attachment of droplets and mitochondria to intermediate filaments may provide the elements necessary to regulate the transport of cholesterol by movement of droplets to mitochondria or vice versa. In canine interstitial cells, filaments (5 nm in diameter) of unknown composition were reported to occur near lipid droplets suggesting that these filaments could be involved in cholesterol transport (Connell and Christensen, 1975). The fact that both adrenal and Leydig cells show the same cellular organization for cholesterol transport is consistent with the fact that steroid synthesis appears to be organized in a similar fashion in all steroidogenic cells.

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