LOCALISATION OF A 40kDA PROTEIN IN RAT STEROID PRODUCING CELLS IDENTIFIED BY A MONOCLONAL ANTIBODY

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Objective. To localize (by the light and electron microscopy) and partially characterize the antigen recognized by the Mab 4E6 in rat ovaries.

Methods. Monoclonal antibody (4E6) against a rat ovarian granulosa cell antigen was prepared and identified the 40kDa protein specific for rat steroid producing cells. The localization of this antigen was studied by light and electron microscopic immunocytochemistry.

Results. The immunocytochemical observation suggested that the recognized antigen was localized in granulosa and thecal cells in all stages of follicular development. The intensity of immunostaining was found to depend on the developmental stage. In granulosa and thecal stage (health follicle) Mab 4E6 binding mollecule was localized on the membranes of rough endoplasmic reticulum (RER) and on the surface of lipid droplets in close association with the RER. In atretic follicles we established that the final destination of the visualized antigen is in structures which we refer as the autofagic vacuoles in close contact with the steroidogenic organelles. In addition, we observed Mab 4E6 binding molecule in the cytoplasm of luteal cells, Leydig cells and adrenocortical cells.

Conclusions. The results indicate that the 40kDa antigen may be common to all of rat steroidogenic organs. Our results suggest that the 40kDa protein may be associated with the processes governing steroidogenesis and/or follicular development.

Key words: Monoclonal antibody – 40 kDa antigen – Immunocytochemistry – Ovarian follicle – Steroidogenesis

Development of the ovarian follicle is a highly integrated process which involves a series of sequential events in which the recruited follicle acquires its structural and functional properties as it matures, ovulates and became luteinized. It has been reported that the events taking place during follicular development result from the interactions between the different steroidogenic compartments and these enable the dominant follicle to acquire eminence over other follicle which undergo atresia (Dorrington et al. 1984; Adashi 1992; Wang et al. 1993).

The concept of follicular regulators was developed from the observation that steroid synthesized by the follicle could act locally on the cells within the same follicle. The synthesis of steroids is regulated by both FSH and LH (Richards 1980). In addition to these gonadotropic control mechanisms, intraovarian and most likely also intrafollicular mechanisms play an important role in follicular maturation as well as in steroidogenesis. Several steroidal and nonsteroidal local factors are known to modulate the growth and steroidogenesis (Hsueh et al. 1984). However, the identification of specific proteins involved in these processes, the identification of the source of these proteins (i.e. either derived from the circulation or by local production) and their physiological significance are not completely understood.

One possible approach to examine the intraovarian regulatory mechanisms is the use of immunological studies. Cell specific molecules are of particu-

lar importance in the processes of growth, proliferation and steroidogenesis. Characterization of antigens using monoclonal antibodies (Mabs) in one of the approaches to identify specific molecules involved in these processes.

Using gonadotropin stimulated rat granulosa cells as immunogen we accumulated a library of Mabs against rat developmentally associated antigens (Russinova et al. 1994). Monoclonal antibody from a hybridoma line 4E6 reacted immunocytochemicaly with an antigen found in all ovarian steroidogenic compartments – granulosa cells, thecal cells and luteal cells. In addition, Mab 4E6 also labeled Leydig cells in rat male gonads and rat adrenocortical cells.

The aim of this work was the light and electron microscopic localization as well as the partial characterization of the antigen recognized by the Mab 4E6 in rat ovaries.

Materials and Methods

The preparation and characterization of the specificity of the antibody, used in this study, have been described previously (Russinova et al. 1994). Briefly, Balb/c mice were immunized with granulosa cells ($5x10^6$) collected from rat ovaries after pregnant mare serum gonadotropin (PMSG) stimulation. Fusion was performed using a P3x63-Ag8.653 myeloma line. On immunocytochemical screening a Mab 4E6 was found to react with an antigen localized in all rat ovarian steroidogenic compartments. In addition Mab 4E6 was found to react in a similar staining pattern with rat testes and adrenocortical gland but did not react with other tissues – lung, liver and kidney. This Mab was of IgG type.

Immunocytochemistry: avidin-biotin technique (ABC). Paraffin sections from adult rat ovaries fixed in Bouin's solution were processed for light immunocytochemistry using avidin – biotin peroxidase technique of Hsu et al. (1981). In this procedure methanol peroxide solution was used to block endogenous peroxidase activity and normal rabbit serum was used to block nonspecific binding of the secondary antibody. Sections were incubated with Mab 4E6 (hybridoma supernatant) for 18h at 40C then rinsed with phosphate buffered saline (PBS) and incubated for 60 min with biotinilated anti-mouse. IgG (Vector-Burlingam) diluted 1:250 in PBS. After rinsing in PBS avidin – biotin – peroxidase conjugate (Vector-Burlingam) diluted 1:250 in PBS was applied for 60 min. Visualization of the binding sites was accomplished with 3, 3' – diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris – HCl – buffered saline (ph 7.6) 0.01% H_2O_2, dehydrated and coverslipped. In addition, cryosections from rat adrenal gland and testes were also processed for ABC procedure using Mab 4E6 as immunocytochemical probe.

Immunocytochemistry of granulosa cell culture was performed using immunofluorescence microscopy Briefly, immature 25 – day – old Wistar rats were injected with 8 IU of PMSG. 48h later granulosa cells were isolated from ovaries and plateled in Dulbeco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum. Granulosa cells were grown on glass coverslips for 48h and additional 18h in serum free media then washed with PBS and fixed in methanol for 6 min at -200C. Primary antibody was added for 1h at room temperature. The cells were then rinsed in PBS to wash away unbound antibody. The same incubation and washing procedures were used for FITC – labeled goat anti – mouse IgG secondary antibody (Sigma Chemical Co., USA) diluted 1:30 with PBS. After extensive washing the coverslips were mounted on microscope slides in 90% glycerol in PBS and viwed with a Zeiss epifluorescence microscope.

Controls for light microscope observations. Control sections from rat ovaries were incubated either with normal mouse serum or with control antibody (Mab 3D8) which bound a 76kDa antigen localized in rat granulosa cells but did not react with thecal, luteal, adrenal and Leydig cells (Russinova et al. 1998) and Mab 3C2 which bound an acrosomal component (Russinova et al. 1998). Additional controls was performed using Mab 4E6 preabsorbed with protein extract obtained from the cells utilized for immunization.

Immunoelectron microscopy. Electron microscopic immunocytochemical studies were performed on 301m thick frozen sections from rat ovaries fixed with a solution containing 4% paraformaldehide, 0.1% glutaraldehide and 0.15% picric acid in 0.1M phosphate buffer (ph 7.4). The ABC technique was applied as described above. Following visualization of the peroxidase activity the sections were treated

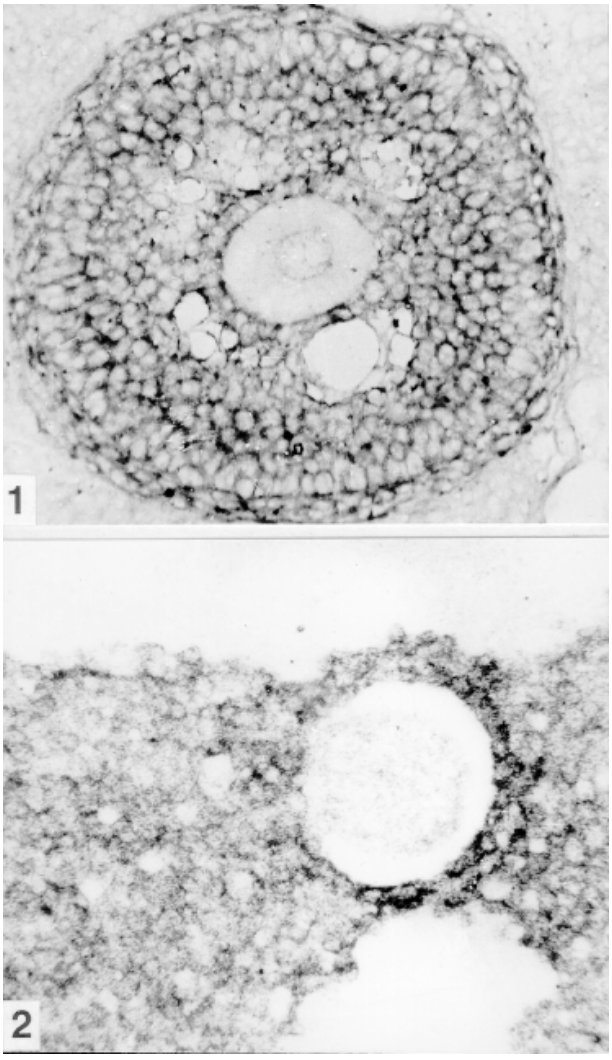

Fig. 1 ABC staining of a rat early antral follicle. The reaction product was observed in the cytoplasm of granulosa and thecal cells, x 200.

Fig. 2 Immunoperoxidase staining of a rat late antral stage. Strong reactivity was observed in the compact region of cumulus oophorus, x 300.

with 1% OsO_4 and embedded in Durcopan by invert consule method. Ultrathin sections were observed without additional counterstaining in a Zeiss EM 109. Controls were performed as follows: (a) the first antibody was omitted ; (b) the ABC procedure was omitted; (c) incubation with Mab 4E6 preabsorbed with protein extract obtained from the cells utilized for immunization.

Polyacrylamide gel electrophoresis (SDS-PAGE). The granulosa cell pellet obtained from the

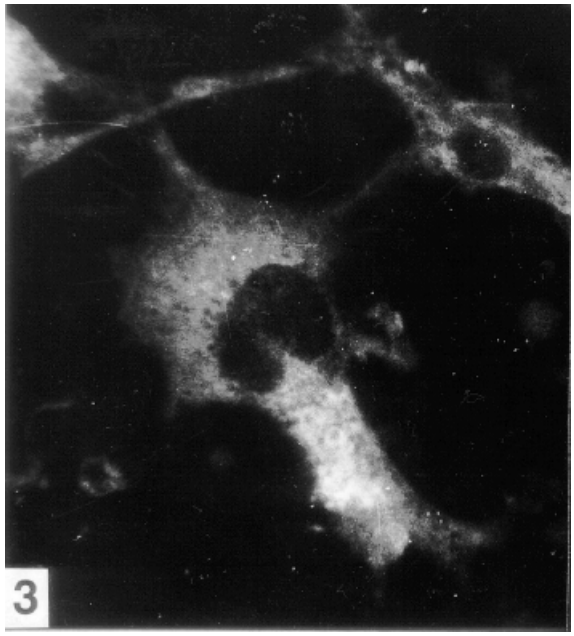

Fig. 3 Immunofluorescence of granulosa cell culture. Fine reticular network throughout the cytoplasm of granulosa cells was observed, x 640.

cells utilized for immunization was homogenized on ice in PBS containing 1% Triton X-100 and following protease inhibitors. 1mM PMSF and 51g/ml each of benzamidine HCI, aprotinin and lenpeptin. Following extraction the mixture was centrifuged at 20000xg for 5min at 40C and the supernatant was collected. The protein extract was suspended in sample buffer in a boiling water bath for 5min.

SDS – PAGE was run on a 12.5% separating gel (ph 8.7) with stacking gel (ph 6.8) according to Laemmly (1970). Molecular mass was calculated from the position of markers that were subjected to electrophoresis in parallel lates.

Immunoblotting proteins separated by SDS – PAGE were transferred on to nitrocellulose membranes. The transferred proteins were stained using Ponceau S (Sigma, France) as described by Harlow and Lane (1988), destained by washing with Tris – buffered salins (TBS, 10mM Tris – Cl, ph 8, 100mM NaCl) and the blots were cut vertically in 5mm wide strips. To prevent nonspecific protein binding the nitrocellulose strips were blocked for 2h at 370C with 3% bovine serum albumin (BSA). The nitrocellulose membranes were then incubated with Mab 4E6 (appropriate dilution with TBS – 1% BSA) overnight

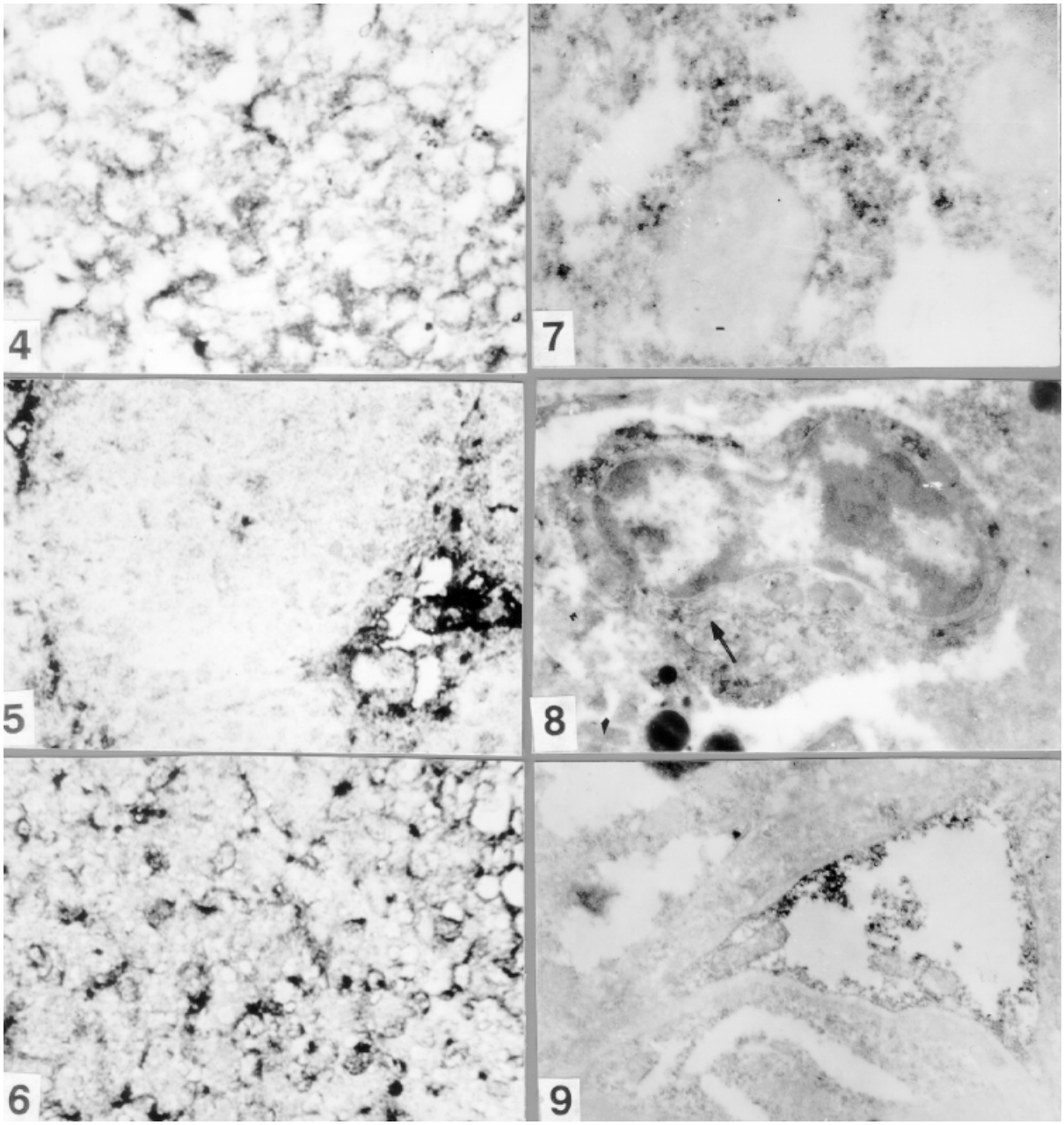

Fig. 4, 5, 6 ABC staining of the cytoplasm of luteal cells (4) Leydig cells (5) and adrenocortical cells (6), x 300, x 160, x 200 (respectively).

Fig. 7, 8, 9 Immunoelectron micrographs of portions of granulosa cell cytoplasm. The reaction product was observed on the membranes of RER and on the surface of lipid droplets in nonatretic follicle (7). Labeled RER in thecal cells (arrow) (8). In luteal cells the reaction product was observed on the cell surface and on the ribosomes in close association with the smooth endoplasmic reticulum (9), x 30000, x 7000, x 22000 (respectively).

at 40C and for 1h at room temperature. After washing with TBS 0.05% Tween 20, the antigen – antibody complex was visualized with avidin – biotin peroxidase method.

Parallel strips treated with non – immune serum or control antibody (Mab 3D8) served as negative controls. Additional negative control was performed with Mab 4E6 preabsorbed with protein

extract obtained from the cells utilized for immunization.

Results

Immunocytochemistry. Under the light microscope immunoreactivity in rat ovaries was found in the cytoplasm of granulosa cells and thecal cells in all stages of follicular development (Fig. 1,2). The intensity of immunostaining increased with the degree of follicular development. As growth progressed the reaction became stronger both in granulosa and thecal cells. The strongest reactivity was observed in early and late antral stages. An especially strong reactivity was observed in the compact region of stratified cuboidal epithelial cells (cumulus oophorus)) in late antral stage (Fig. 2). Oocytes were in all stages negative. In cultured granulosa cells immunofluorescence revealed a fine reticulare network, that spread throughout the cytoplasm (Fig. 3).

In atretic follicles the reaction was observed both in granulosa and thecal cells but the strength of the immunoreaction decreased with the degree of atresia (not shown). In addition, we found that Mab 4E6 also labeled in similar staining pattern the luteal cells, the Leydig cells in rat male gonads, and adrenocortical cells in zona glomerulosa and zona fasciculata of rat adrenal glands (Fig. 4,5,6).

Control experiments omitting the primary antibody or using the control antibody and preabsorbed Mab 4E6 were negative (not shown).

The subcellular localization of the reaction product was examined by immunoelectron microscopy. In granulosa and thecal cells (nonatretic follicles) the reaction product occurred on the membranes of the rough endoplasmic reticulum (RER) (Fig. 7,8). The reaction product was often found concentrated on the surface of lipid droplets in close association with the RER (Fig. 7).

In atretic follicles the reaction product was also observed on the membranes of RER but the strength of the reaction decreases with the degree of atresia. The antigen recognized by the Mab 4E6 in granulosa sells of atretic follicles was observed also in structures with a specific morphological appearance, which we tentatively defined as the autofagic vacuoles. In general, the clustering of autofagic vacuoles that we seen are mostly spherical structures and contain numerous labeled reaction product, lipid droplets, small labeled vesicles and small labeled RER membranes (Fig. 10,11,12). The autofagic vacuoles are often in close contact with steroidogenic organelles as lipid droplets, mitohondria and labeled RER. In controls no labeling was observed in autofagic vacuoles (Fig. 13).

In luteal cells numerous reaction product was observed on the cell surface and on the ribosomes in close association with the smooth endoplasmic reticulum (Fig. 9).

Antigen identification. Granulosa cell protein extract obtained from the cells utilized for immunization was subject to SDS-PAGE and immunoblotting using the ABC procedure. A single band of an apparent molecular mass of 40 kDa was detected on blot (fig.14, lane 2). Control blots omitting the primary antibody or using the control Mabs and preabsorbed Mab 4E6 were negative (not shown).

Discussion

By using a variety of immunological probes many characteristics of cell communication during follicular development were identified. Maruo and Okada (1987) described antigens localized in granulosa cells and oocytes and proposed that granulosa cell products are transferred to oocytes during development. Iron et al. (1994) using Mab X80 identified oocyte stage specific antigen. In recent years we have obtained several kinds of Mabs specific for antigens in rat gtanulosa cells and oocytes (Russinova et al. 1994) and for a 59kDa antigen common to all of rat steroid producing cells (Russinova et al. 1995).

In the present study we utilized a monoclonal antibody (4E6) prepared against a granulosa cell antigen and identify a 40kDa protein also specific for ovarian steroid producing cells. In addition, we found that Mab 4E6 also labeled the Leydig cells and adrenocortical cells. These results indicate that the recognized antigen may be common to all of the rat steroidogenic organs. The binding of Mab 4E6 to all steroid producing cells is not surprising. In our previous work using Mab 5G5 as immunological probe we identified a 59kDa antigen also

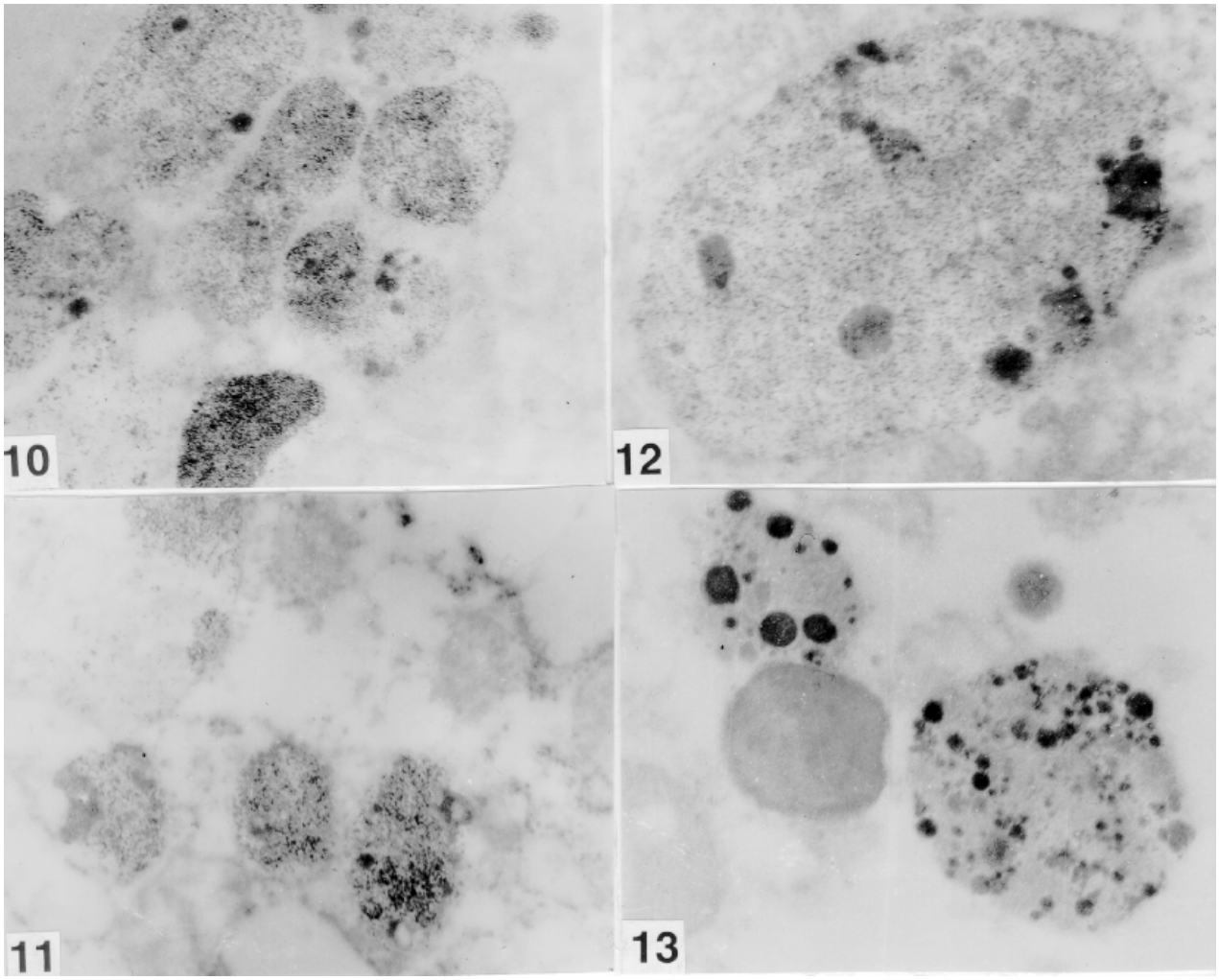

Fig. 10, 11, 12 Immunoelectron micrographs of portions of granulosa cells in an atretic follicle. The reaction product was observed in autophagic vacuoles, x 22000, x 30000, x 49000 (respectively).

Fig. 13 In control section no labeling was observed in autophagic vacuoles, x 30000.

specific for rat steroid producing cells. However, the antigen recognized by the Mab 5G5 was localized on the cell surface of steroid producing cells (Russinova et al. 1993, 1995). The Mab 5G5 binding molecule has similar molecular weight to the steroidogenesis inducing protein (SIP) (Shafiq et al. 1992) which protein exerts stimulatory effect on steroidogenesis by ovarian and Leydig cells as well as by adrenal cells .

In the present study using immunoelectron microscopical observation of health follicle we established that the reaction product was localized on the RER of granulosa and thecal cells. Using in vitro conditions we observed expression of the recognized antigen in the granulosa cell cytoplasms. On the basis of these results it can be expected that the 40kDa antigen is most probably produced by the granulosa and thecal cells. The degree of follicular maturation and the position of granulosa cells within the follicle appear to modulate their capacity for production of the 40kDa antigen.

In the atretic follicles using Mab 4E6 we established that the final destination of the 40kDa protein is in structures which we refer as the autofagic vacuoles. It is known that cell growth is governed by a delicate balance between the synthesis and deg-

radation of proteins. The degradation of endogenous proteins occurs primary through autophagic mechanisms (Mortimore et al. 1989). Autophagy has been implicated in the degradation of normal proteins in response to nutrient deprivation; in the cellular remodeling that occurs during differation metamorphosis and transformation, toxicity or death (Mortimore et al. 1989; Dunn, 1990). Autophagy is a general mechanism occurring in many cell types whereby intercellular organelles and cytosol are first sequestered away from the remaining cytoplasm and then degraded within the lysosomes (Dunn 1990). The evidence suggests that autophagic vacuole formation can be regulated by a variety of physiological (e.g., amino acids and hormones) effectors (Reunanen et al. 1985; Mortimore et al. 1989). On the other hand the growth and development of an ovarian follicle proceed uninterruptedly until the follicle either ovulates or undergoes a degenerative process known as atresia (Richards 1980). The results obtained by Francis et al. (1991) demonstrate an intimate association between apoptotic like events and dying granulosa cells and thus support the possibility that appoptosis is involved in the induction of follicular atresia. The atretic alterations occurred initially in granulosa cells and subsequently in the thecal cell layer (Tsafriri and Brow 1984). It is possible that substances which can stimulate appoptosis in granulosa cells may be responsible for the induction of atresia in whole follicles. Because follicles are exposed to a large number of substances (i.e. growth factors, cytokines, hormones, etc) in vivo, it is possible that the presence or withdrawal of these factors either alone or in combination, modulates the induction of appoptosis in granulosa cells and consequently atresia in the entire follicle. However, despite the overwhelming occurrence of atresia in the ovary, the cellular and molecular events underlying this phenomenon remain poorly understood. In this aspect labeling of the autofagic vacuoles with Mab 4E6 could be used as a marker for the onset of atresia.

The results reported in this study provide no evidence concerning the physiological role of the visualized antigen. On basis of immunocytochemical localization we can only speculate that the 40kDa protein may be associated with the processes governing steroidogenesis and/or follicular development. Understanding the organization and control of steroidogenesis may elucidate one of the major purples in follicular development and Mab 4E6 may be useful in this endeavor.

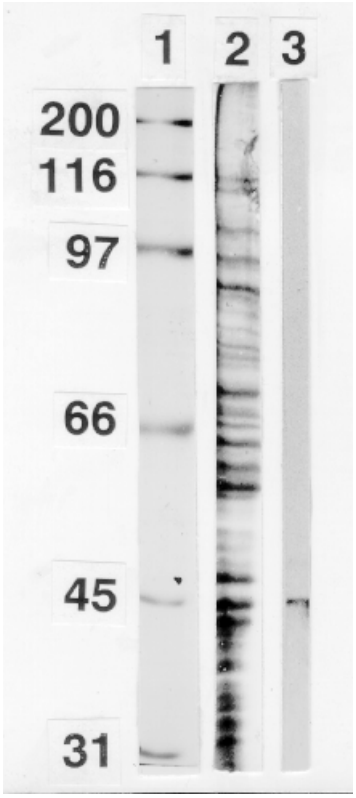

Fig. 14 Characterization of the 4E6 antigen. Lane 1 – the positions of molecular mass markers in kDa; Lane 2 – protein spectrum obtained from the cells utilized for immunization; Lane 3 – nitrocellulose replica of the same gel as shown in lane 1 labeled with Mab 4E6; the band with molecular size of approximately 40kDa was stained.

For these findings the 40 kDa antigen recognized by the Mab 4E6 is worth further investigation for understanding its function during follicular development.

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