Morphofunctional Modifications Associated with the Inhibition by Estradiol of MtTF₄ Rat Pituitary Tumor Growth

Jacqueline Trouillas, Yves Morel, Marie-Odile Pharbaz, Geneviève Cordier, Christian Girod, and Jean André

Laboratoire d’Histologie et Embryologie, Faculté de Médecine Alexis-Carrel, Rue Guillaume Paradin, 69372 Lyon Cedex 08 [J. T., C. G.]; Laboratoire de Biochimie, INSEM U 80, Hôpital Edouard-Herriot, 69372 Lyon Cedex 08 [G. C.], France

Hôpital Debrousse, INSERM U. 34, 29 Rue Soeur Bouvier, 69322 Lyon Cedex 05 [Y. M., M-O. P., J. A.]; and Centre de Cytofluorométrie, Faculté de Biologie Humaine, INSEM U 50, Hôpital Edouard-Herriot, 69372 Lyon Cedex 08 [G. C.], France

ABSTRACT

The MtTF₄ pituitary tumor has been induced in Fischer rats by chronic estrogen administration. Recently, we reported that sustained pharmacological treatment of Fischer rats with 17β-estradiol inhibited the growth of the MtTF₄ tumor transplanted s.c. The present work describes the associated morphofunctional changes occurring in the tumor during 17β-estradiol inhibition. It is shown that a 7-day 17β-estradiol treatment resulted in an increase of the surface area of cells, nuclei, nucleoli, Golgi complexes, and rough endoplasmic reticulum and an increase in the number of euchromatin-rich nuclei. Flow cytometry analysis of DNA distribution suggested that estradiol affects the cell progression through the early S phase. The ratio of RNA to DNA increased significantly, reflecting cell hypertrophy. Moreover, there was a significant increase in tumor prolactin concentration and a marked enhancement in the intensity of the immunocytochemical reaction with rat prolactin antiserum. On the other hand, cell mitoses were dramatically decreased.

These morphofunctional changes indicate that the inhibition of the tumor growth by estradiol is accompanied by an evolution of the tumor cell population towards a more differentiated state. However, it cannot be decided whether 17β-estradiol induces a shift from a proliferative state to a differentiated state or whether 17β-estradiol treatment results in a selection of a subpopulation of tumor cells that are slow growing and more differentiated.

INTRODUCTION

17β-Estradiol stimulates the multiplication of some normal or tumoral target cells, namely epithelial cells. Estradiol administration in vivo induces cell multiplication in anterior pituitary (23), uterus (28), mammary gland (31), and mammary tumors (18) of numerous animal species and the in vitro incubation of cell lines derived from human breast cancer with estradiol-increased cell mitoses (21). In addition, circumstantial evidence strongly suggests that estradiol is also able to inhibit cell multiplication. Estrogen induces dimethylbenzanthracene mammary tumor regression (13). Daily estradiol injections initially stimulate and subsequently inhibit the DNA synthesis in response to further estrogen treatment in pituitary and uterus (30). Added to cell culture medium, estradiol inhibits the multiplication of numerous kinds of cells, including human pituitary adenoma cells (33) and MCF₂, or ZR breast tumor cells (1, 21). Since the estradiol concentrations used were higher than 10⁻⁷ M, the biological significance of these observations remained questionable. Howev

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2 To whom requests for reprints should be addressed.

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Materials and Methods

Animals, Tumors, and Hormone Treatments. Two- to 3-month-old male F344 rats were given s.c. injections of a suspension of MtTF₄ tumor cells as described previously (25). Three weeks later, the rats were divided in 2 groups. One group served as control (n = 8), while the other one (n = 15) was implanted with a Silastic tubing (Catalogue No. 602-285; Dow Coming Corp., Midland, MI; 1 cm long) filled with estradiol. In another experiment, the Silastic tubing was filled with DES or DHT. The rats were dispatched in the different groups according to the volume of their tumor: they were chosen in order to get tumors of similar weight at the time of decapitation. Control and test rats were killed 7 days after the beginning of hormone treatment. Tumors were removed immediately and processed for biochemical and/or histological studies.

Processing of Tumors for Morphological Studies and Counting of Mitoses. For light microscopy (histological observations and immunofluorescence studies), tumor fragments were fixed in the Bouin-Hollande HgCl₂ fluid, embedded in Paraplast, and cut at 4 μm of thickness. Histological observations were performed on slides stained with Masson's trichrome, Herlant's tetrachrome, and PAS/orange G; the number of mitoses/thousand cells was determined after examination of at least 1000 cells/tumor. The areas of nuclei and nucleoli were determined, after carmine/alun staining, with a Quantimet 720 (Cambridge Instrument); 50 measures/tumor were done on fields taken randomly in different areas of the tumor. Immunofluorescence studies were done by the Weller and Coon method, as described previously (14). The following rabbit antisera were used: 17β-hydroxy-3-keto-5α-androstane; GH, growth hormone; PRL, prolactin; ACTH, adrenocorticotropic hormone; PAS, periodic acid-Schiff.

The abbreviations used are: DES, diethylstilbestrol; DHT, 17β-hydroxy-3-keto-5α-androstane; GH, growth hormone; PRL, prolactin; ACTH, adrenocorticotropic hormone; PAS, periodic acid-Schiff.
kindly provided by M. P. Dubois were used at 1/100 dilution: anti-human GH; anti-rat PRL; and anti-17,39-ACTH. The specificity of the antisera has been reported previously (8). Sections from tumors of control and estradiol-treated rats and sections from pituitary of control rats were treated with the same antisera in the same manipulation. It has been checked that no positive reaction was observed when antisera were incubated previously with an excess of antigen or when slides were incubated with nonimmune serum. The percentages of immunopositive cells were estimated with a Zeiss epiilluminated fluorescent microscope by the same person and were given only for comparative purpose.

For electron microscopy, tumor fragments, taken randomly, were fixed in 4% glutaraldehyde in cacodylate buffer (pH 7.4, 100 m"e) and postfixed in 2% osmium tetroxide. They were embedded in Araldite. The ultrathin sections, cut with a Reichert OMM 3 ultramicrotome, were contrasted with uranyl acetate and lead citrate and finally examined with a JEOL-type JEM 7 electron microscope.

Processing of Tumor for Flow Cytometry. Small pieces of tumor were washed with RPMI 1640 medium (Seromed, Blockrom KG, Berlin, Germany) and treated with 0.1% collagenase (type I; 152 units/mg; Worthington Biochemical Corp., Freehold, NJ), 0.01% DNase I (from bovine pancreas, 800 Kunitz units/mg; Sigma Chemical Co., St. Louis, MO), and 5 mM MgCl₂ in RPMI 1640 medium. The cell suspension was filtered through a polyester gauze (30 μm) and centrifuged at 500 × g for 5 min at 200 × g. The pellet was washed twice with RPMI 1640 medium; resuspended in the same medium; layered on top of 3 layers of 5, 30, and 60% Percol (Pharmacia Fine Chemicals, Uppsala, Sweden) prepared with RPMI 1640 medium; and centrifuged for 20 min at 400 × g. Tumor cells were located in the 30% layer. RBC and nuclei were found at the bottom, while membranes and damaged cells were found at the top.

Tumor cells were washed twice with RPMI 1640 medium. Viability, determined by the trypsin blue exclusion method, was higher than 90%. Cells were fixed with absolute ethanol and stained with ethidium bromide in conditions described previously (10), except that the RNase treatment was performed for 1 hr and the pepsin treatment was omitted. Analyses were carried out in the Cytofluorograph 50 H (Ortho Instruments, Westwood, MA), and data were monitored with a LYSIS system developed in our laboratory. With the staining procedure used, the intensity of red fluorescence was proportional to the cell DNA content. Doublets were distinguished from cells in the G₀ phase by measuring the peak and the area of the red fluorescence pulses. DNA distributions were established from 20,000 cells. In these studies, no attempt was made to calculate the relative proportion of cells in the different phases; only the changes in the shape of the histograms were recorded.

Processing of Tumor for Biochemical Studies. Frozen tumors, kept at -70°C, were pulverized with a stainless steel mortar and pestle in liquid nitrogen. Powdered samples were homogenized in Tris-HCl (pH 8.9, 50 mM)/disodium tetraborate (12.5 mM)/EDTA (5 mM) cold buffer and were allowed to stand for 5 hr in an ice-bath. Aliquots of the homogenates were sonicated before measuring DNA by the method of Burton (6) with calf thymus DNA as standard, RNA by the orcinol method (7) with yeast tRNA as standard, and protein by the method of Lowry et al. (22) with bovine serum albumin as standard. The homogenate remains were centrifuged for 20 min at 25,000 × g at 4°C. PRL and GH were measured in the supernatant by radioimmunoassay using the materials and the protocols supplied by the Pituitary Hormone Distribution Program (National Institute of Child Health and Human Development). The GH cross-reaction with PRL antisera was 0.6%, and the PRL cross-reaction with GH antisera was 1.6%. The results were expressed in ng equivalents of rat PRL/RP₂ or GH/RP₂.

Miscellaneous. All data are given as the mean ± S.D., and significances were determined by Student's t test.

RESULTS

Sustained high-dose treatment with estradiol for 7 days of the rats bearing MtTF₄ tumor resulted in marked modifications of the naked eye appearance and of the consistency of the tumor. The tumors of estradiol-treated rats were firmer and less hemorrhagic than were the tumors of control rats.

Light Microscopy. In control tumors, cells were either organized in round sheets centered by a capillary in nodule-like figures or arranged diffusely. Connective stroma was poorly developed, and large areas of necrosis were often present (Fig. 1). The cells were small, round, and monomorphic (Fig. 2); agranular with Herlant's tetrachrome staining; and PAS negative.

Following estradiol treatment, the general architecture of the tumor was markedly modified. The necrotic areas tend to decrease. The cells were arranged in sheets or cords lined by a well-developed connective tissue (Fig. 4), and they were enlarged, agranular, and PAS negative. The evident increase of the nucleus and nucleolar areas (cf. Fig. 5 to Fig. 2) was confirmed by quantimetry as shown in Table 1. Simultaneously, estradiol treatment reduced dramatically the number of mitoses/thousand cells (Table 1; Figs. 2 to 5).

Immunocytochemistry. In control tumors, PRL immunoreactive cells (Fig. 3) varied between 5 and 100% from area to area in a given tumor and from one tumor to another. In a given cell, the fluorescence was weak and homogeneous. The most striking and constant consequence of estradiol treatment was the enhancement of the fluorescence intensity/cell which frequently capped the nucleus (Fig. 6). Nevertheless, the intensity of the reaction varied from cell to cell. The percentage of positive cells was rather higher than in control tumors; more than 50% of positive cells were found in 12 of 15 treated tumors and only 4 of 8 control tumors.

No immunofluorescence was detected in cells of control or estradiol-treated tumors using anti-human GH serum. The absence of immunoreactivity was not due to the type of antisera used, since it allowed the detection of GH cells in normal rat

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.1000 cells</th>
<th>Range n</th>
<th>Area (sq μm)</th>
<th>Range n</th>
<th>Area (sq μm)</th>
<th>Range n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>41.4 ± 7.0*</td>
<td>30-52 8*</td>
<td>24.3 ± 2.3</td>
<td>22.0-28.7</td>
<td>1.35 ± 0.18</td>
<td>1.12-1.67</td>
</tr>
<tr>
<td>Estradiol-treated</td>
<td>15.7 ± 4.3*</td>
<td>9-23 15</td>
<td>46.9 ± 3.5*</td>
<td>44.1-53.9</td>
<td>3.73 ± 0.75*</td>
<td>2.56-4.78</td>
</tr>
</tbody>
</table>

*Mean ± S.D.

Number of data are 5.
pituicy gland. ACTH-immunoreactive cells were observed in some control and estradiol-treated tumors. The immunofluorescence was always very weak compared to the fluorescence intensity observed in the pituitary gland, and no variation was observed under estradiol treatment.

Electron Microscopy. In control tumors (Fig. 7), the cells appeared monomorphous and undifferentiated. They were rich in free ribosomes. The rough endoplasmic reticulum was very limited. The secretory granules were very rare and often marginally situated. The Golgi complex was either absent or very limited.

The most characteristic modifications produced by estradiol treatment (Fig. 8) were: (a) an increase of the rough endoplasmonic reticulum which was sometimes vesicular or dilated; (b) a rise of the number of the secretory granules, although the cells remained sparsely granulated; round or polymorph granulations, often located in the Golgi area or lining the cell membrane; small granulations, measuring from 100 to 150 nm in diameter; (c) an increase of the Golgi complex area; (d) the appearance of some exocytosis figures; and finally (e) an increase of the number of euchromatin-rich nuclei. These modifications were consistent in the 15 estradiol-treated tumors examined in comparison with 8 control tumors, but there were variations from cell to cell in the same tumor.

Flow Cytometry Analysis of DNA Distribution. DNA distribution of tumor cells from control or estradiol-treated rats are illustrated in Chart 1. In control tumors, the majority of cells are found in the major G0-G1 peak, the plateau represents cells in the S phase, and the minor peak corresponds to the G2 phase after exclusion of doublets. This pattern is similar to that observed with an asynchronously growing cell population. Following estradiol treatment, the major peak is clearly enlarged towards higher DNA content, suggesting a block near the early S phase. The coefficient of variation is 13.1% in estradiol-treated versus 6.4% in control tumors. Similar results were observed in 4 additional experiments with different rats.

Biochemistry. The control tumors contain PRL and GH. Following estradiol treatment, the PRL content increased markedly, from 39.2 ± 5.6 to 131.0 ± 38.9 μg/g of tumor (p < 0.01 in Experiment 1 of Table 2). The GH content was not significantly modified. The protein or RNA contents per g of tumor were not modified by estradiol treatment, whereas the DNA concentration was significantly reduced from 8.85 ± 0.65 to 4.80 ± 0.46 mg/g of tumor. Consequently, the RNA/DNA ratio increased sharply.

Table 2

<table>
<thead>
<tr>
<th>PRL (μg/g)</th>
<th>GH (μg/g)</th>
<th>Proteins (mg/g)</th>
<th>RNA (μg/g)</th>
<th>DNA (μg/g)</th>
<th>RNA/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44b</td>
<td>11.1</td>
<td>115</td>
<td>6.00</td>
<td>9.25</td>
</tr>
<tr>
<td>44</td>
<td>8.0</td>
<td>100</td>
<td>5.06</td>
<td>9.22</td>
<td>0.54</td>
</tr>
<tr>
<td>33</td>
<td>6.4</td>
<td>110</td>
<td>4.45</td>
<td>7.88</td>
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<tr>
<td>36</td>
<td>6.9</td>
<td>100</td>
<td>5.22</td>
<td>9.06</td>
<td>0.57</td>
</tr>
<tr>
<td>(39.2 ± 5.6)c</td>
<td>(8.1 ± 2.1)</td>
<td>(106 ± 7)</td>
<td>(5.18 ± 0.63)</td>
<td>(6.85 ± 0.65)</td>
<td>(0.57 ± 0.04)</td>
</tr>
<tr>
<td>Estradiol</td>
<td>176</td>
<td>24.0</td>
<td>150</td>
<td>6.1</td>
<td>5.46</td>
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<tr>
<td>80</td>
<td>9.9</td>
<td>116</td>
<td>4.32</td>
<td>2.22</td>
<td>1.02</td>
</tr>
<tr>
<td>105</td>
<td>12.2</td>
<td>112</td>
<td>6.89</td>
<td>4.90</td>
<td>1.36</td>
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<tr>
<td>141</td>
<td>12.0</td>
<td>121</td>
<td>5.36</td>
<td>4.92</td>
<td>1.09</td>
</tr>
<tr>
<td>157</td>
<td>11.2</td>
<td>106</td>
<td>5.72</td>
<td>4.54</td>
<td>1.25</td>
</tr>
<tr>
<td>(131.0 ± 38.9)d</td>
<td>(13.8 ± 5.7)e</td>
<td>(121 ± 17)f</td>
<td>(6.03 ± 1.43)g</td>
<td>(4.90 ± 0.46)h</td>
<td>(1.24 ± 0.19)i</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.6</td>
<td>14.1</td>
<td>135</td>
<td>7.12</td>
<td>10.87</td>
</tr>
<tr>
<td>23.6</td>
<td>36.8</td>
<td>139</td>
<td>8.60</td>
<td>12.99</td>
<td>0.66</td>
</tr>
<tr>
<td>Estradiol</td>
<td>64.0</td>
<td>12.3</td>
<td>126</td>
<td>7.98</td>
<td>4.58</td>
</tr>
<tr>
<td>60.0</td>
<td>5.8</td>
<td>110</td>
<td>9.23</td>
<td>4.58</td>
<td>2.01</td>
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<tr>
<td>DES</td>
<td>86.9</td>
<td>12.6</td>
<td>120</td>
<td>9.09</td>
<td>3.68</td>
</tr>
<tr>
<td>58.2</td>
<td>41.6</td>
<td>114</td>
<td>8.65</td>
<td>4.04</td>
<td>2.19</td>
</tr>
<tr>
<td>DHT</td>
<td>16.1</td>
<td>19.1</td>
<td>140</td>
<td>8.06</td>
<td>11.38</td>
</tr>
<tr>
<td>16.3</td>
<td>13.1</td>
<td>135</td>
<td>6.74</td>
<td>10.28</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* Content/g of fresh tumor.
* Values located on the same vertical line were obtained in the same tumor, except for those values in parentheses.
* Mean ± S.D.
* p < 0.01 versus control.
* p < 0.001 versus control.

Content/g of fresh tumor.
Values located on the same vertical line were obtained in the same tumor, except for those values in parentheses.
Mean ± S.D.
p < 0.01 versus control.
*p < 0.001 versus control.

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under estradiol treatment.

Similar morphological (not shown) and biochemical (Table 2) alterations were observed after DES treatment, while DHT was inactive on both parameters.

**DISCUSSION**

This work describes changes occurring in the MtTF4 tumor after an estradiol treatment which is known to inhibit the growth of this tumor (20, 25, 27). Morphological, biochemical, and immunocytochemical evidence indicates that the inhibition of tumor growth, documented by a dramatic decrease of the mitotic index, is accompanied by an evolution of the tumor cell population towards a more differentiated state.

One of the most striking consequences of the estradiol treatment, as revealed by light microscopy, is the cell hypertrophy (Figs. 1 to 6). This hypertrophy is the most probable explanation for the decrease of the DNA content/g of tumor, since it has for consequence a marked decrease of the tumor cell density (cf. Fig. 1 with Fig. 4). Other possibilities cannot be definitely excluded, but they would play a minor role only. (a) The artifactual degradation of DNA during the extraction process of tumors from estradiol-treated rats is unlikely, since the tumor protein and RNA contents are not altered. (b) There is no significant change of the mean value of the DNA content/cell as determined by fluorimetric assay in dispersed cells. (c) The flow cytometry analysis of DNA distribution suggests that estradiol treatment increases the number of cells in early S phase and excludes definitely that such a treatment decreases the DNA content below that of G0-G1 control cells. (d) The mitotic index is so low in control tumors that its further reduction from 41 to 15% following estradiol treatment cannot substantially modify the biochemical properties of the cell population.

Although estradiol treatment stimulates the synthesis and the secretion of PRL in the pituitary gland, substantial evidence strongly suggests that the increase of the tumor PRL content results from local alterations of its synthesis, degradation, and/or secretion and not from a contamination of the tumor tissues by the circulating PRL from a pituitary source. (a) The intensity of the tumor intracellular PRL immunoreactivity is increased under estradiol treatment. (b) We reported earlier that similar estradiol treatment enhances the PRL mRNA translation activity in the tumor (25). (c) If we assume that plasma represents 5% of the tumor volume and knowing that the plasma PRL concentration in tumor-free rats treated for 7 days with estradiol is 1187 ± 236 ng/ml, the contamination by the circulating PRL would be in the range of 50 ng/g of tumor, i.e., less than 1% of the measured PRL. (d) The effect of estradiol treatment on the tumor PRL content displays some specificity, since neither GH nor ACTH contents are increased.

The comparison of the consequences of estradiol administration in MtTF4 tumor (this paper) with those reported by others in anterior pituitary is helpful to direct further studies to determine the mechanism by which estradiol stimulates or inhibits the cell multiplication. The effects of *in vivo* estradiol treatment in rat anterior pituitary have been extensively studied. Some modifications are qualitatively similar to those reported here, such as the stimulation of PRL synthesis (23), the hypertrophy of PRL cells, and the enlargement of nucleoli, Golgi complexes, and rough endoplasmic reticulum (3). However, some differences are evident. (a) The enlargement of nuclei looks more limited in pituitary than in MtTF4. (b) In the pituitary, the number of mitoses is markedly increased (17). The RNA/DNA ratio remained constant under estradiol treatment (19), leading to the suggestion that cell hyperplasia played the major role in the estradiol stimulation of pituitary growth. (c) The translation activity of PRL mRNA, the number of PRL secretory granules, and the PRL content/g of tissue were higher (in basal conditions and after estradiol treatment) in pituitary than in tumor. For example, the PRL concentration in the tumor was 20- to 50-fold higher in pituitary than in tumor. (e) Two mRNAs coding for M, 26,000 and 36,000 proteins were shown to be induced or increased by estradiol treatment in the tumor but not in the pituitary (25). (f) The concentration of type D2 dopamine receptor was dramatically reduced in the tumor but not in the pituitary (2). Our working hypothesis is that estradiol induces, specifically in the tumor, the synthesis of growth-inhibiting factors.

Another way to find specific features of cells of which growth is inhibited by estradiol is to compare the current morphological aspects of the MtTF4 with those of the transplantable tumor, at different periods of its evolution, in relation to its estradiol dependency or estradiol responsiveness. Cells of the DES-dependent pituitary tumor were shown to be similar to normal PRL cells (9, 11). When the tumor became autonomous, *i.e.*, able to grow in rats not treated by estrogen, the mitotic activity was higher than in DES-dependent tumor, and the cells looked less differentiated (11, 28, 29). This was documented mainly by the presence of a poorly developed rough endoplasmic reticulum and a limited number of secretory granules. However, the growth of the autonomous tumor remained sensitive to DES (9) or estradiol (24, 25). According to the effects of DES on tumor growth, Clifton and Furth (9) described 2 kinds of tumors: one inhibited and the other stimulated by DES. Interestingly, these 2 tumors share similar features in the optic and the electron microscopy (9). Consequently, additional features are needed to distinguish among the autonomous tumors in which growth is stimulated from those in which growth is inhibited by estradiol. However, what appeared clearly is that the MtTF4 tumors in which growth is inhibited by estradiol are characterized by a large population of poorly differentiated cells and a relatively high mitotic index.

Two hypotheses may be proposed to explain the association of the inhibition of tumor growth and the morphofunctional changes observed in the tumor after estradiol treatment. (a) Estradiol treatment results in a selection of a subpopulation of tumor cells that are slower growing and more differentiated. (b) Estradiol induces a shift of cells from a proliferative, poorly differentiated state into a more differentiated, slow-growing state. At the present time, we cannot decide which hypothesis is correct.

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**REFERENCES**


Figs. 7 and 8. Ultrastructural features of MtTF2 tumors, control (Fig. 7) and 17α-estradiol treated (Fig. 8).

Fig. 7. Cells are small, monomorphic, and undifferentiated. Free ribosomes are numerous, but rough endoplasmic reticulum and sacculi are rare. Golgi complex and granulations are absent. × 10,000.

Fig. 8. Fragment of 2 large cells with a well-developed rough endoplasmic reticulum, 2 Golgi complexes, small granulations lining the cell membranes, and liposomes. The nuclei are enriched in euchromatin. × 10,000.
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