Light and electron microscopic immunocytochemistry on the localization of 17α-hydroxylase/C17,20-lyase (P450c17) in the rat placenta

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Abstract : The rat placenta is the primary source of androgens during the second half of pregnancy. Androgens are converted to estrogens in the ovaries and contribute to the maintenance of normal pregnancy. We immunocytochemically characterized the cellular and subcellular localization of cytochrome P450 of 17α-hydroxylase/C 17,20-lyase (P450c17), an enzyme responsible for androgen synthesis, in the rat placenta. We also observed the fine structure of the placenta by electron microscopy. The rat placenta had a different structure from the primate, and contained four zones : labyrinth, basal zone, decidua basalis, and metrial gland. The labyrinth had three trophoblastic layers and fetal endothelium, and P450c17 immunoreactivity was homogeneously localized in the three trophoblastic layers but not in the fetal endothelium. In the basal zone, various types of trophoblasts were observed, and the immunoreaction was localized in small basophilic cells and giant cells. The intensity of staining was heterogeneous among these cells. The decidua basalis showed no immunostaining. Subcellular localization of the enzyme was in the cytoplasm, but not in the nucleus or mitochondria. The present study demonstrated a steroidogenic potency in both the labyrinth and the basal zone, although it was shown only in the basal zone in previous studies. J. Med. Invest. 44 : 155-162, 1998

Key Words : *17α-hydroxylase/C17, 20-lyase, rat, placenta, immunocytochemistry, electron microscopy*

INTRODUCTION

The placenta becomes the principal source of androgens during the second half of pregnancy in the rat (1). This is correlated with the appearance of the enzyme, P450c17, responsible for androgen synthesis (2, 3). Androgens serve as substrates for the enzyme, aromatase, to synthesize estrogens that are essential for normal pregnancy (4).

The rat placenta has a different structure from the primate (5, 6), and contains four zones : labyrinth, basal zone, decidua basalis, and metrial gland (7, 8). The labyrinth is the zone in which both maternal vascular channels and fetal vessels are found. The basal zone, in which fetal mesenchyme is absent and only maternal vascular channels are found, is situated between the labyrinth and the decidua basalis. Previous biochemical and histochemical observations demonstrated that the basal zone is the principal site of steroidogenesis (9, 10, 11). Only a histochemical study reported that P450c17 immunoreactivity was localized in giant cells, a subpopulation of the basal-zone cells (12). However, the basal zone occupies only 15% of the mature placenta by weight, and the giant cells are a small number of population in the basal zone (8). Because the subcellular structure is not significantly different among the trophoblast phenotypes and the placenta produces large amount of steroids during pregnancy (2, 8, 13), the enzyme may also be contained in other populations of the rat placental cells. In the present study we have examined immunocytochemically the cellular and subcellular localization of P450c17 using the silver-gold intensification method to improve the sensitivity for immunoreaction (14). We have focused on the rat placenta on the 18th day of pregnancy, because it has a mature structure and the expression of the enzyme in the placenta is known to peak on this day of pregnancy (2, 8).

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MATERIALS AND METHODS

Animals

Three pregnant rats of Wistar strain (Japan SLC, Shizuoka, Japan) were maintained on a 12-h light-dark cycle at 20°C and fed lab chow and water ad libitum.

Tissue preparation

On the 18th day of pregnancy, rats were anesthetized with ether and slaughtered by cervical dislocation. The uterus was opened and the conceptuses were removed. Then, the placentae were separated from the decidual tissue and immersed in the fixative of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, overnight at 4°C. The tissues were soaked in 10, 15 and 20% sucrose in 0.01 M phosphate-buffered saline (PBS), pH 7.4, successively. The specimens were frozen and cut in 8-μm-thick sections using a cryostat. The frozen sections were thaw-mounted on glass slides coated with chrome alum gelatin and air-dried for about 1 h. The sections were provided for transmission electron microscopy (TEM) and immunocytochemistry.

TEM

For general electron microscopic observations, the sections were postfixed in 1% osmium tetroxide for 1 h at room temperature, rinsed with 10% sucrose in distilled water, and stained with a saturated solution of uranyl acetate for 30 min at room temperature. Then, they were dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were cut on a Reichert Ultracut E, stained with uranyl acetate and lead citrate, and examined in a Hitachi H-500 electron microscope.

Immunocytochemical procedure

The sections were rinsed with 0.01 M PBS for 10 min and soaked in 0.2% Triton X-100 in 0.01 M PBS for 20 min. Endogenous peroxidase was blocked by an incubation with 3% hydrogen peroxide in distilled water for 20 min at room temperature, and endogenous avidin/biotin binding was blocked with avidin D, followed by biotin (Vectastain, Vector, USA). The sections were then incubated with the antiserum against P450c17 (provided by Dr. Kominami, Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima, Japan) (15, 16) diluted 1 : 4,000 in 0.01 M PBS containing 1% normal goat serum (NGS) overnight at 4°C, with biotinylated goat anti-rabbit IgG diluted 1 : 100 in 0.01 M PBS containing 1% NGS for 1 h at 32°C, and then with streptavidin-peroxidase (Histofine, Nichirei, Japan) for 1 h at 32°C. The immunoreaction was visualized in 50 mM TRIS-HCl buffer, pH 7.6, containing 0.01% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide, and examined by light microscopy.

After the peroxidase reaction, some of the sections were treated with the silver-gold intensification method (14), and then processed for electron microscopy as mentioned above. The thin sections were examined under the electron microscope without uranyl acetate or lead citrate staining.

As the control, some sections were incubated with normal rabbit serum in place of the antiserum against P450c17.

RESULTS

Light microscopic observations

The rat placenta on the 18 th day of pregnancy consisted of labyrinth, basal zone, and decidua basalis (Fig.1). The labyrinth occupied two thirds of the placenta

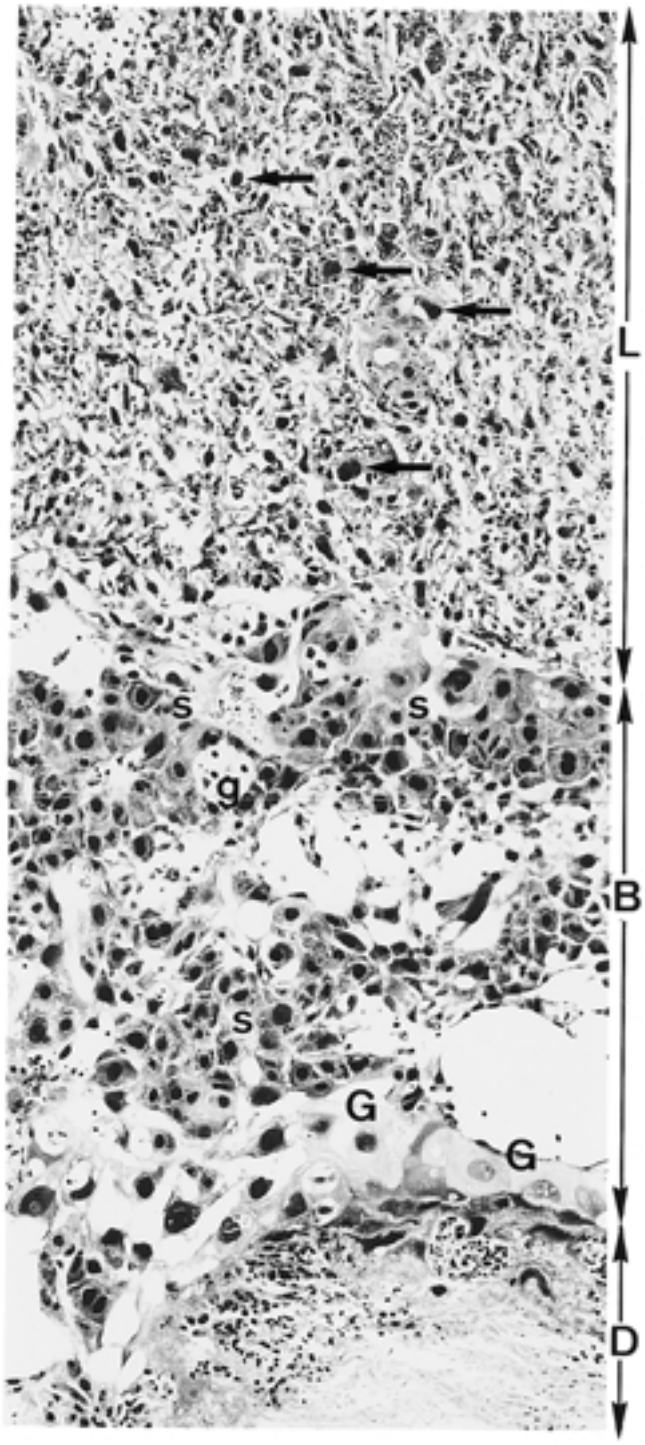

Fig.1. Light micrograph of the fetal rat placenta on the 18th day of pregnancy. The rat placenta consists of labyrinth (*L*), basal zone (*B*), and decidua basalis (*D*). In the labyrinth (*L*) fetal vessels and maternal vascular channels are found. The labyrinthine trophoblasts show round nuclei and basophilic cytoplasm (*arrows*). The basal zone (*B*) is situated between the labyrinth and the decidua basalis, and there only maternal vascular channels are found. In this zone, small basophilic cells (*s*), glycogen cells (*g*), and giant cells (*G*) are observed. Hematoxylin-eosin staining. x120.

and contained both fetal vessels and maternal vascular channels. The labyrinthine trophoblasts had round nuclei and basophilic cytoplasm (Fig.1). The basal zone had only maternal vascular channels and contained small basophilic cells, glycogen cells, and giant cells (Fig.1).

The immunoreactivity for P450c17 was observed in the trophoblastic epithelium of the labyrinth, and in the small basophilic cells and the giant cells of the basal zone (Fig.2, 3). In the labyrinth, the immunoreaction was relatively weak and distributed homogeneously in the trophoblastic epithelium. In the basal zone, the reaction was observed more intense compared with the labyrinth, and the intensity of immunoreaction was heterogeneous among the small basophilic and the giant cells regardless of the cell type. The immunoreaction was localized in the cytoplasm and not in the nucleus. The decidua basalis was immunonegative. There was no immunoreaction in the control sections incubated with normal rabbit serum (data not shown).

Electron microscopic observations

The fine structure of the rat placenta was observed by TEM, and the cellular and subcellular localization of P450c17-immunoreactivity was confirmed by immunoelectron microscopy using the preembedding and the silver-gold intensification method.

In the labyrinth, trophoblastic epithelium had a trilaminar structure : layer 1, lying adjacent to the maternal vascular channel ; layer 2, intermediate in position ; layer 3, in contact with endothelial cells of the fetal vessels through basement membrane (Fig.4 a). The labyrinthine trophoblast had abundant rough endoplasmic reticulum, round mitochondria with lamellar or tubular cristae, a round nucleus, and a clear nucleolus (Fig.5 a). The

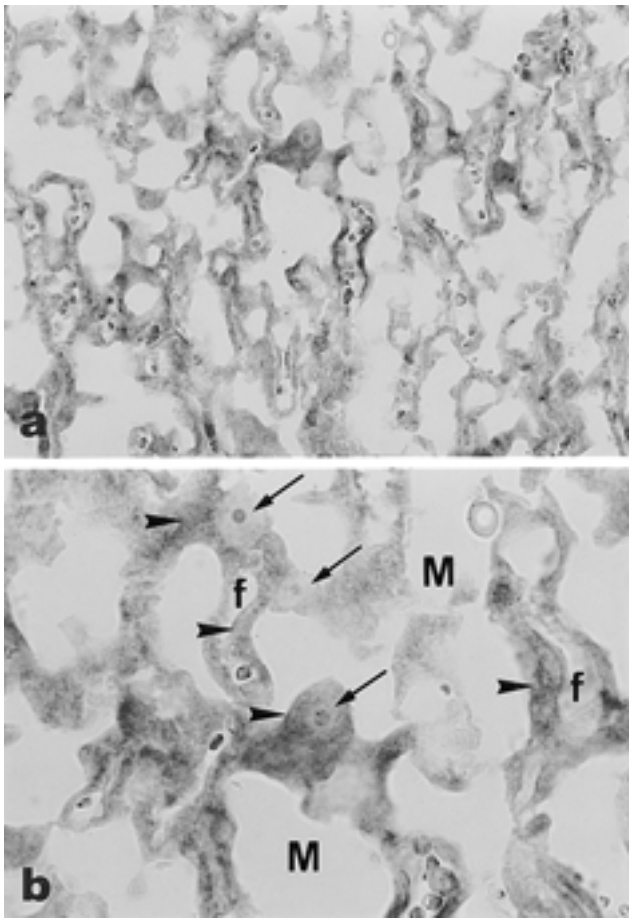

Fig.2. **a**, **b** Immunostaining of P450c17 in the labyrinth of the rat placenta on the 18th day of pregnancy. **a** Trophoblastic epithelium is entirely immunopositive. **b** Immunoreactivity is localized in the laminar and perinuclear cytoplasm of the trophoblasts (*arrowheads*). Their nuclei are immunonegative (*arrows*). *M* maternal vascular channel, *f* fetal vessel. **a** x210, **b** x420.

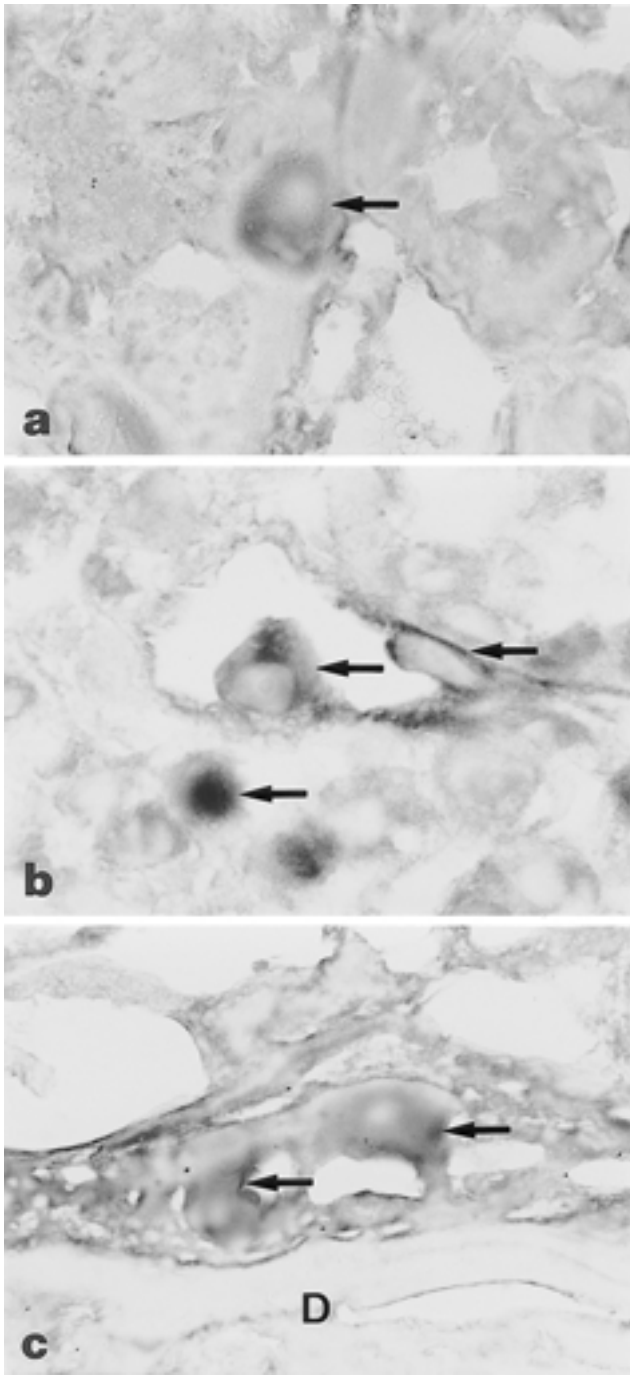

Fig.3. **a-c** Immunostaining of P450c17 in the basal zone of the rat placenta on the 18th day of pregnancy. **a**, **b** Small basophilic cells show the immunoreactivity in their cytoplasm (*arrows*). **c** Giant cells, that lie between the small basophilic cells and the decidua basalis (D), are immunopositive (*arrows*). x420.

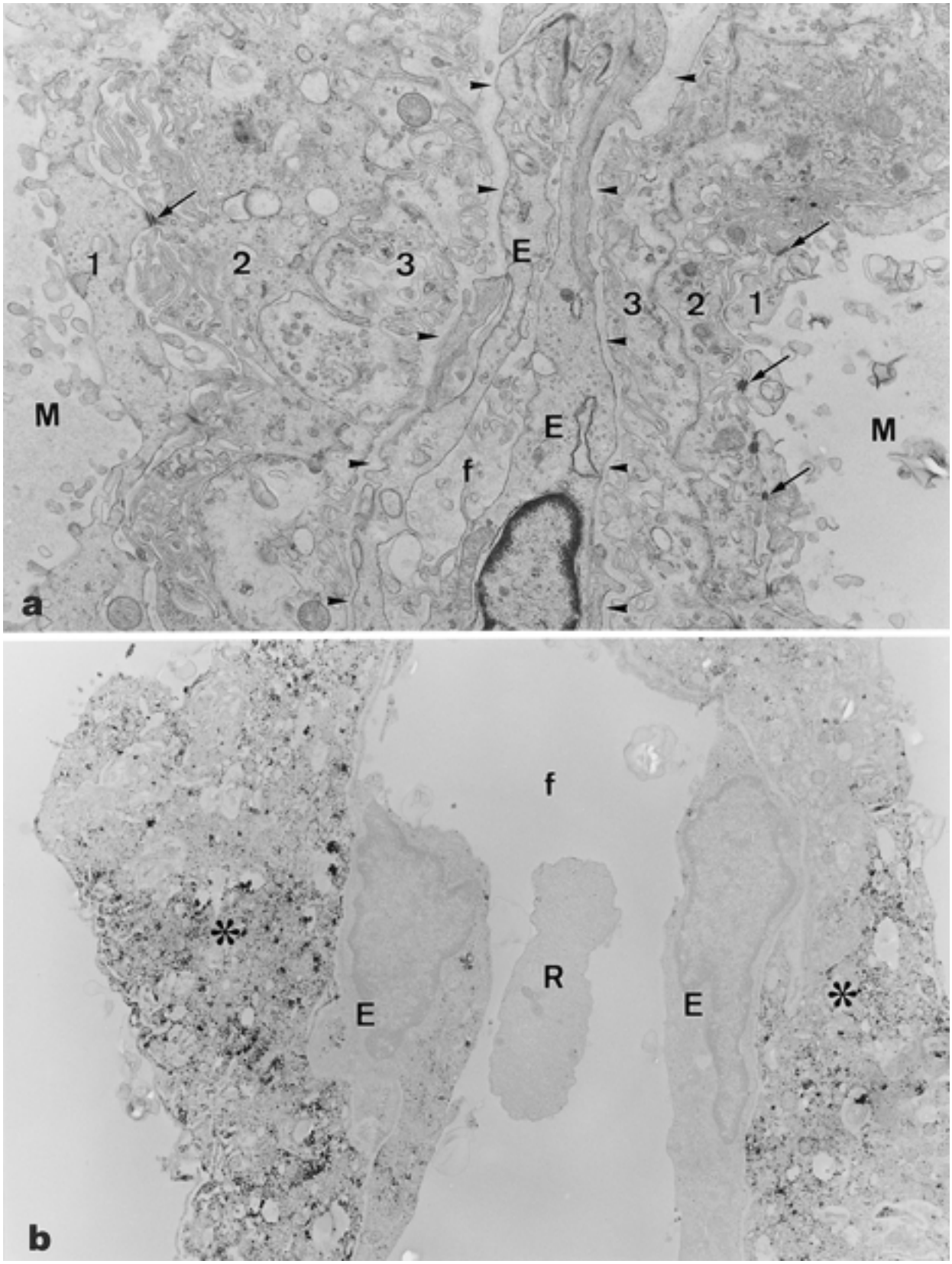

Fig.4. a, b Electron micrograph of the layers of trophoblastic epithelium in the labyrinth. **a** The ultrastructure of the layers. The labyrinthine trophoblasts show trilaminar structure as follows : layer 1 (*1*), lying adjacent to the maternal vascular channel (*M*) ; layer 2 (*2*), intermediate in position ; layer 3 (*3*), in contact with endothelial cells (*E*) of the fetal vessel (*f*) through basement membrane (*arrowheads*). **b** Immunocytochemistry of P450c17 in the layers. Silver grains, which correspond to the location of the DAB endproducts, are homogeneously present in the trilaminar trophoblastic epithelium (*asterisks*). The endothelial cells (*E*) of the fetal vessel (*f*) and a fetal erythrocyte (*R*) are immunonegative. *Arrows* indicate desmosomes between the layer 1 and 2 trophoblasts. **a** x10,000, **b** x7,000.

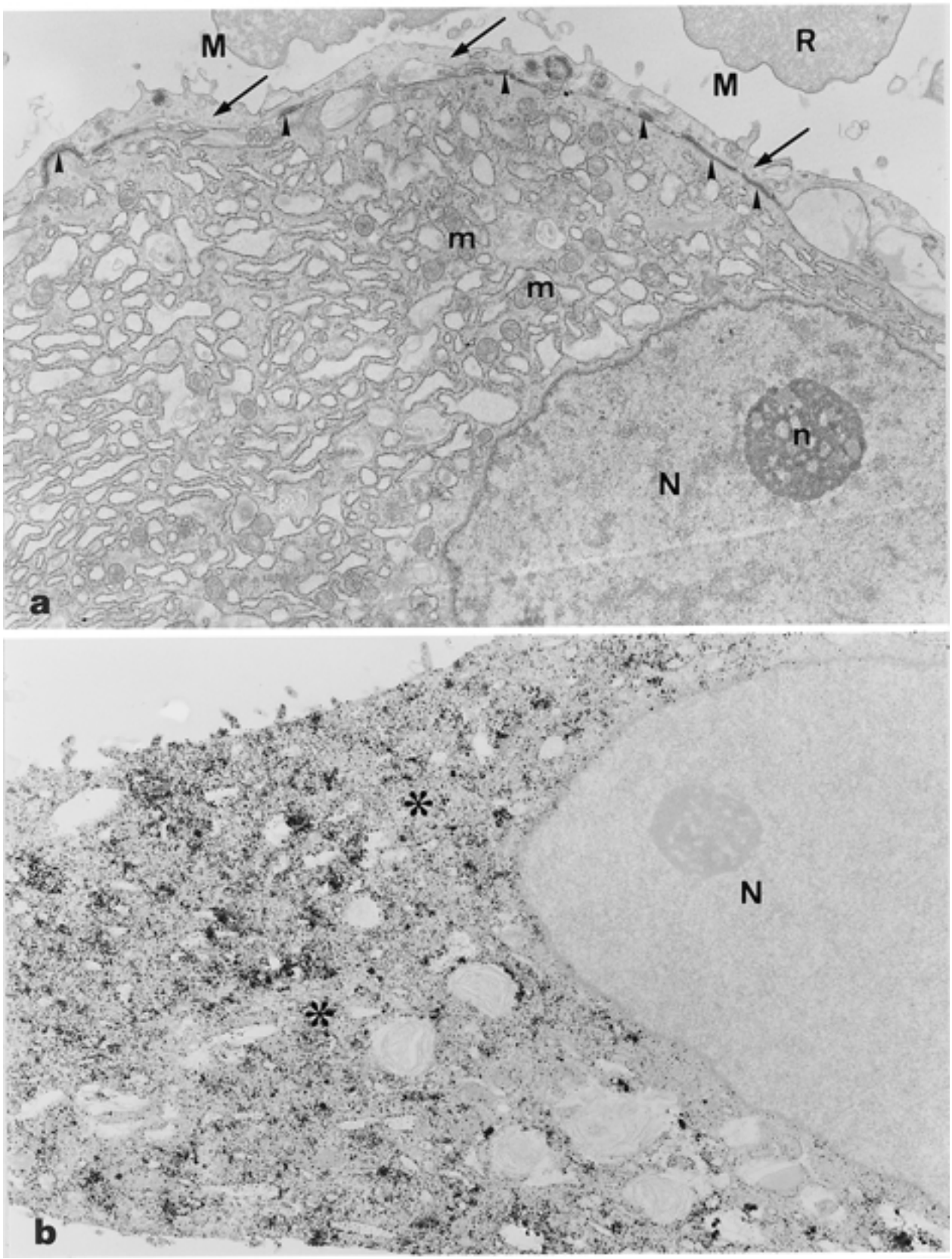

Fig.5. a, b Electron micrograph of the perinuclear area of the labyrinthine trophoblast in layer 2. **a** The ultrastructure of the perinuclear area. The trophoblast has abundant rough endoplasmic reticulum, round mitochondria (*m*) with lamellar or tubular cristae, a round nucleus (*N*), and a clear nucleolus (*n*). Many desmosomes are present in the junction between the layer 1 and 2 trophoblasts (*arrowheads*). The layer 1 trophoblast has a thin cytoplasmic layer (*arrows*) and adjoins the maternal vascular channel (*M*). **b** Immunocytochemistry of P450c17 in the perinuclear area of the labyrinthine trophoblast. Immunoreaction products are localized in the cytoplasm (*asterisks*), but not in the nucleus (*N*). *R* maternal erythrocyte. **a** x8,000, **b** x11,000.

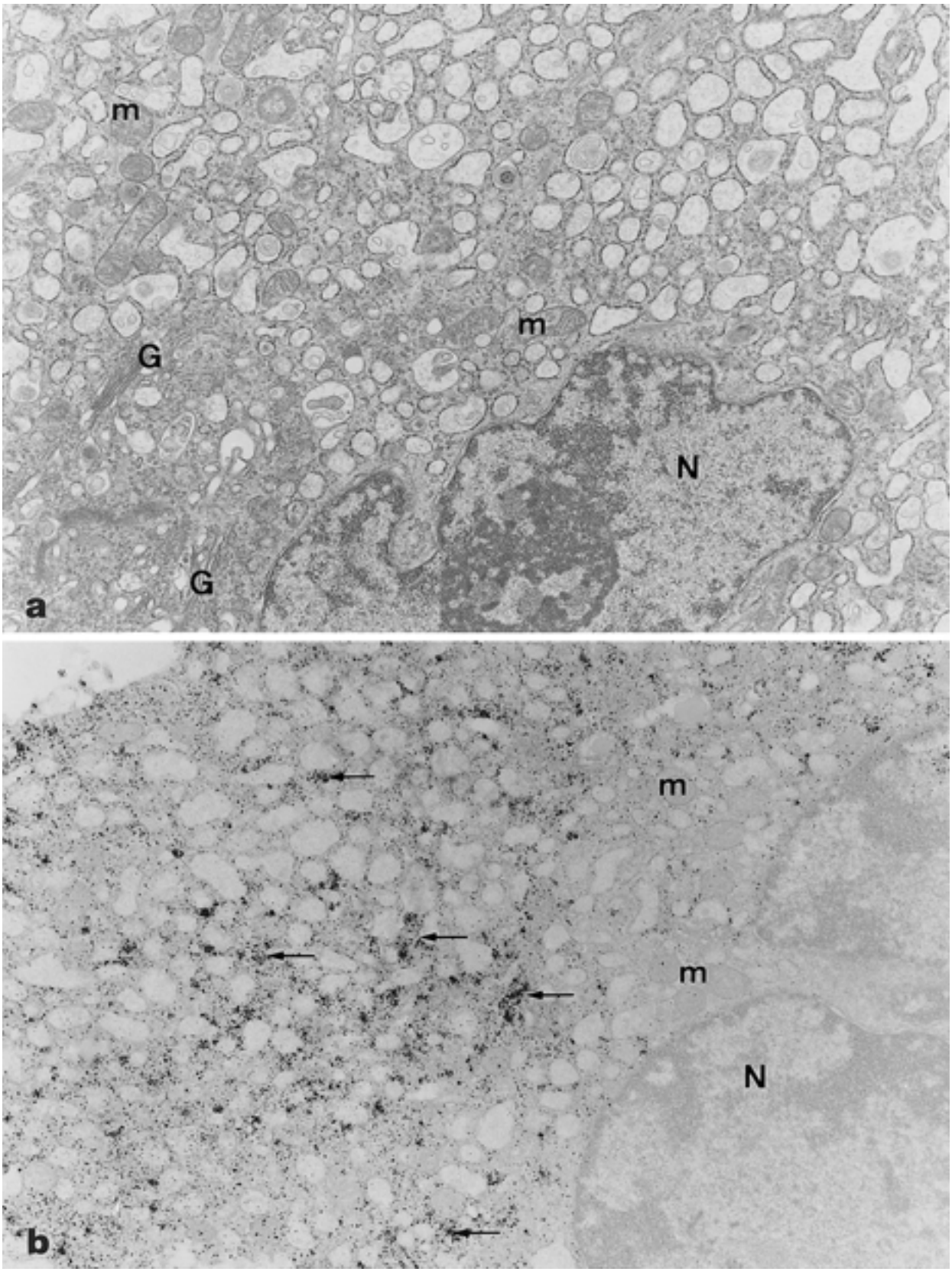

Fig.6. a, b Electron micrograph of the giant cell in the basal zone. **a** The ultrastructure of the giant cell. The giant cell has well-developed rough endoplasmic reticulum, oval or elongated mitochondria (*m*) with lamellar or tubular cristae, abundant Golgi complexes (*G*), numerous free ribosomes, and an irregular-shaped nucleus (*N*). **b** Immunocytochemistry of P450c17 in the giant cell. The immunoreactivity is localized in the cytoplasm (*arrows*), but not in the nucleus (*N*), mitochondria (*m*) or the lumen of the endoplasmic reticulum. **a, b** x14,000.

immunoreactivity for P450c17 was localized in the trophoblastic epithelium but not in the endothelial cells (Fig.4 b, 5 b).

In the basal zone, the giant cells had well-developed rough endoplasmic reticulum, oval or elongated mitochondria with lamellar or tubular cristae, abundant Golgi complexes, numerous free ribosomal granules, and an irregular nucleus (Fig.6 a). The small basophilic cells had a similar structure (data not shown).

Subcellular localization of the immunoreactivity for P450c17 was in the cytoplasm of the trophoblast, but not in the nucleus, mitochondria or the lumen of the endoplasmic reticulum (Fig.5 b, 6 b).

DISCUSSION

This immunocytochemical study demonstrated P450c17-containing cells in the rat placenta. The immunoreactivity was weak in the trophoblastic epithelium of the labyrinth, and intense in the trophoblasts of the basal zone. These results suggest that steroidogenic potency is heterogeneously localized in the rat placenta. The same findings have been reported as the following : The production of progesterone was about ten times higher in the isolated basal zone than in the whole placenta (10). The significant activity of the C17,20-lyase and 17α-hydroxylase was observed in the basal zone but it was insignificant in the labyrinth (11). Only an immunocytochemical report by Johnson showed that the enzyme was localized in the giant cells of the basal zone (12). The present immunohistochemical study has shown that the enzyme was localized not only in the basal zone but also in the labyrinth. The discrepancy between our results and Johnson's may be due to the difference of antibodies used or sensitivity of immunostaining methods. Although the immunoreactivity in the labyrinth was weak compared with the basal zone, the labyrinth occupied two thirds of the mature placenta, and hence the amount of steroids produced in the labyrinth might be large in total.

This study has also demonstrated the heterogeneous immunoreactivity among the basal-zone trophoblasts. This heterogeneity may be due to the difference of the enzyme content among the cells or to the unequal accessibility of the antibody.

The enzyme P450c17 is responsible for androgen synthesis. Because the adrenal glands of rodents do not express P450c17 and do not produce androgens (17), the major sites of androgen biosynthesis in female rodents are the ovary and the placenta (1). The androgens serve as the substrates for aromatase to synthesize estrogens, which are crucial for the normal progression of pregnancy (4). In the pregnant rat, aromatizable androgen synthesis is developmentally regulated : Before the 11th day of pregnancy, the principal source of the androgens is the ovary. As gestation advances, the ovary loses its ability to produce androgens, and the placenta evolves as the principal source of androgens (1, 2). Since the rat placenta is deficient in the aromatase activity (10), androgens produced in the rat placenta, are converted into estrogens in the ovary, especially in the corpus luteum, which maintains the aromatase activity during gestation (18).

In other species such as the cow (19, 20), pig (21), and sheep (22), the placenta contains both P450c17 and aromatase, and hence estrogens are synthesized in the placenta. In the human, rhesus monkey, baboon and horse, the placenta expresses aromatase but does not express P450c17 (6, 23). Estrogen synthesis in the placenta depends on a source of androgens from the fetus ; the fetal adrenal glands in the case of the primate, and the gonadal interstitial cells in the case of the horse.

The structure of the placenta varies remarkably across species (5, 6). The rat placenta has a different architecture from the primate, and consists of labyrinth, basal zone, decidua basalis, and metrial gland (Fig.1) (7, 8). In the labyrinth, the trophoblastic epithelium is trilaminar (Fig.4), and its fine structure has been previously characterized (7, 8). The layer 1 trophoblasts, which adjoin the maternal blood, are not syncytial, but the layer 2 and 3 trophoblasts are syncytial. The attachment of the layer 1 and 2 is extremely loose, and many desmosomes are present. The layers 2 and 3 are in close opposition, and occasionally united by desmosomes. The ultrastructural features of these three layers are similar except that free ribosomes are abundant in layer 1, smooth vesicular components of the endoplasmic reticulum are pronounced in layer 2, and lipid droplets are more commonly found in layer 3 than in the other layers (8). These morphological characteristics may represent functional differences among the layers, but P450c17-immunoreactivity was homogeneously localized in these three layers (Fig.4, 5).

There are small basophilic cells, glycogen cells and giant cells in the basal zone, in which fetal mesenchyme is absent and only maternal vascular channels are observed (Fig.1) (8). The small basophilic cells are characterized by their abundant endoplasmic reticulum and by the numerous ribosomal granules. Similar to the small basophilic cells, the giant cells are characterized by the extensive development of the rough endoplasmic reticulum (Fig.6). They are considered to be derived from the small basophilic cells (8). The small basophilic cells and the giant cells were immunopositive for P450c17. The glycogen cells are characterized by extensive accumulations of glycogen granules in their cytoplasm and their features occasionally appear empty due to the disappearance of glycogen during tissue preparation(8). These cells also belong to the trophoblastic cell lineage, but these cells showed no immunoreactivity for P450c17.

In this study the subcellular localization of P450c17 in the rat placenta was demonstrated in the cytoplasm but not in the nucleus, mitochondria, or the lumen of the endoplasmic reticulum (Fig.5,6). The silver grains were distributed homogeneously in the cytoplasm, but these reactions may be partly attributed to the diffusion of DAB chromogen or an excess of silver intensification. The enzyme belongs to the cytochrome P450 species, which are usually membrane-bound (15, 16), and hence the subcellular localization of the enzyme in the placenta may

be in the endoplasmic reticulum.

In summary, this immunocytochemical study has localized P450c17, a key enzyme to produce androgens, in the rat placenta. These results suggest that the rat placenta has a steroidogenic potency in both the labyrinth and the basal zone.

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