Morphological study of pituitary tumorigenesis in transgenic mice induced by hybrid oncogene of the thyrotropin ß-subunit and the simian virus 40 large T-antigen

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Summary. We have created a transgenic mouse, TTP-1, generating anterior pituitary tumors by using the simian virus 40 (SV40) large T antigen gene and human thyrotropin β-subunit gene. To examine characteristics of tumors, histological details were investigated using light and electron microscopies. The main tumor tissues, composed of small chromophobe cells, were located inferior to but clearly separated from the hypothalamus; however, neuron fibers probably derived from the hypothalamus were observed to invade some tumor tissues. Some differentiated endocrine cells occupied the caudal region of the tumor. Immunohistochemically, SV40 large T antigen was expressed in the cell nucleus of the undifferentiated cell area, whereas cells expressing several hormones were mainly distributed in the differentiated cell area. Electron microscopically, the undifferentiated cells were divided into 2 types; electron-dense and -lucent cells, the nuclei of which were composed of obscured nucleoli and many notable invaginations of the nuclear membrane. No intracellular microfilamentous structures were observed. Sometimes it was noted that cytoplasmic processes were connected with gap junctions. In the intercellular spaces, there were neuron fibrous and synapse-like structures. In the differentiated cell area, the cell membranes directly contacting other cells were relatively smooth, and many gap junctions were demonstrated. Secretory granules, which were round and less than 100 nm in diameter, were more electron dense in smaller cells than in larger cells. They were aligned just below the cell membrane. Immuno-electron microscopically, positive reactions for SV40 were observed in the nuclei of the undifferentiated cell area. In the differentiated cell area, most of the secretory granules were labeled by GH. TTP-1 transgenic mice should provide a valuable animal model

for studying the pathogenesis of anterior pituitary tumors.

Key words: Pituitary tumor, SV40 large T, Transgenic mice, Thyrotropin

Introduction

It is well known that hormone-producing organs play several important roles in maintaining body homeostasis. One of the most useful instruments for investigating the hormonal cellular interrelationship is the pituitary gland, because it possesses many types of peptide hormones, and their differentiation for expression is regulated by several factors, for example, Pit-1 protein (Camper et al., 1990; Li et al., 1990). The adenohypophysis, called the center of the endocrine system, synthesizes and releases many hormones such as growth hormone (GH), thyroid-stimulating hormone (TSH), lutenizing hormone (LH), prolactine (PRL) and adrenocorticotropic hormone (ACTH).

Recently, transgenic animal model systems have been used for investigating human hormonal mechanisms by many workers (Fukamizu, 1993; Fukamizu et al., 1993). The results obtained from induced or reconstructed gene expression in experimental animals can contribute to the creation of animal models for human disease. Transgenic mice expressing activated oncogenes are also used as experimental and subclinical models for studying tumorigenesis. Brinster and coworkers (1984) used the large T antigen of Simian virus 40 (SV40) as an oncogene and produced transgenic mice that developed characteristic brain tumors. Additionally, transgenic mice containing a Ren-2 promoter and SV40 large T antigen fusion construct develop renal vascular hypertrophy and hyperplasia associated with a markedly suppressed renal renin mRNA, renal renin content, and

plasma renin concentration (Jacob et al., 1991). Thus, the use of transgenic animals in the study of endocrine systems provides a particularly good model.

In recent years, we have created the transgenic mouse designated TTP-1, which generates anterior pituitary tumors by using the SV40 large T antigen gene and human TSHβ gene (Maki et al., 1994). In previous studies using the TTP-1 transgenic mouse line, unexpectedly, the tumor cells did not produce TSH or other anterior pituitary hormones, whereas they clearly expressed SV40 large T antigen. Histological and immunohistochemical analyses showed that the pituitary tumors of TTP-1 transgenic mice were composed of poorly differentiated pituitary cells. These results suggested that the carried human TSHβ gene region is capable of directing organ-specific expression, but not cell-specific expression. The TTP-1 transgenic mouse should provide a valuable animal model for studying the pathogenesis of anterior pituitary tumors.

In the present study, to obtain additional information about the anterior pituitary tumors generated in a previous study, histological details were investigated by light microscopy and electron microscopy.

Materials and methods

Transgenic mouse line

The transgenic mouse, TTP-1, which carries a hybrid transgene composed of the 5'-flanking region of the TSHβ subunit gene, was established by the Institute for Animal Experimentation, School of Medicine, Hokkaido University, as previously reported (Maki et al., 1994). Briefly, a 4400 bp human TSHβ promoter sequence (XhoI/PvuII fragment) which contained 1109 bp of the 5'-flanking region, 39 bp of exon 1 and 3300 bp of intron 1 (Tatsumi et al., 1988) was linked to an ApaI/EcoRI fragment (94 bp) of a rabbit β-globin intron 1-exon 2 junction containing an acceptor splicing site and an SV40 large T antigen DNA fragment (XbaI/BamHI) (Hanahan, 1985). The constructed gene was microinjected into the pronuclei of fertilized C57BL/6 mouse embryos. They were transplanted into the oviducts of pseudopregnant females as described by Yamamura et al. (1984).

The original TTP-1 transgenic mice were phenotypical dwarfs that developed pituitary tumors, wasted away and died by 7-9 weeks after birth. Therefore, to maintain the transgenic mouse line, the ovaries of female mice were transferred to normal foster females, the ovaries of which were removed beforehand according to the method of Yun et al. (1990). These mice were crossed with normal C57BL/6 males.

Light microscopic procedures

Male C57BL/6 and F2 transgenic mice aged 6 weeks were anesthetized by ether inhalation and sacrificed by cervical dislocation. The fresh pituitary samples were frozen in OCT compound (Miles Lab. Co., USA) with liquid nitrogen. Six-μm frozen sections were fixed in Bouin's solution for 5 min at room temperature, stained with hematoxylin and eosin, or immunostained by the peroxidase-antiperoxidase method using the following rabbit antisera to rat pituitary hormones (Kon et al., 1986): TSH (1:8,000, Institute for Endocrinology, Gumma Univ., Japan); LH β-subunit (1:10,000, Institute for Endocrinology, Gumma Univ., Japan); PRL (1:1,000, Institute for Endocrinology, Gumma Univ., Japan); GH (1:5,000, Institute for Endocrinology, Gumma Univ., Japan); ACTH (1:1,000, Chemicon International Inc., USA) and an anti-human chorionic gonadotropin α-subunit (1:150, Seikagaku Co., Japan). To detect SV40 large T antigen, frozen sections were fixed in acetone, and immunostained by the indirect avidin-biotin complex method (Vectastain, Vector Laboratories Inc., USA) using a monoclonal antibody to SV40 large T antigen (Oncogene Science, Inc., USA). All immunostained sections were slightly counterstained with hematoxylin, dehydrated with graded alcohol, and covered by coverslips. Some pituitary samples were treated by paraffin-embedding and immunohistochemistry.

Electron microscopic procedures

The pituitary tissues were removed, cut into 1 mm cubes and fixed with a 2% glutaraldehyde and 2% paraformaldehyde mixture in 0.1M cacodylate buffer (pH 7.3) for 4 hr at 4 °C. Later they were postfixed with or without 1% osmium tetroxide for 2 hr, dehydrated and embedded in epoxy resin. Some tissues fixed with the 2% glutaraldehyde and 2% paraformaldehyde mixture only were embedded in a commercial acrylic medium, Lowicryl K4M resin (Chemische Werke Lowi Co., Germany). Briefly, samples dehydrated in 100% alcohol were intermediated gradually with a 1:2, 1:1, then a 2:1 resin to alcohol mixture, immersed in pure resin and finally polymerized at -35 °C for 24 hr and later at room temperature for 3-4 days with an ultraviolet polymerizer (Dosaka EM Co. Ltd., Japan). Ultrathin sections obtained from osmium-fixed epoxy resin were double-stained with uranyl acetate and lead citrate, and observed with a JEM-1210 electron microscope (JEOL, Japan) at 100 kV. The sections obtained from non-osmium fixed epoxy resin underwent the following immunoelectron-microscopical procedures on paraffin sheets in a moisture chamber (Kon et al., 1992): 1) rinsing with phosphate-buffered saline (PBS) and treatment with 1% bovine serum albumin for 15-30 min; 2) incubation with rabbit anti-rat GH (1:5,000) or TSH (1:8,000) serum at 4 °C overnight; 3) rinsing with PBS three times; 4) incubation with colloidal gold (15 nm)-labeled protein A complex (E-Y Laboratories Inc., USA) at room temperature for 1 hr; 5) rinsing with PBS and then with distilled water; and 6) double-staining and observation. The sections obtained from the Lowicryl K4M-embedded tissues were immunostained with anti-

SV40 large T antigen antibody and colloidal gold (15 nm)-labeled antimouse immunoglobulin G (E-Y Laboratories Inc., USA). Control sections were incubated with normal rabbit serum, murine ascitic fluid or PBS.

Results

Gross anatomically, the pituitary glands enlarged by tumorigenesis in TTP-1 transgenic mice showed hemorrhage with a reddish-brown color and could be easily removed from the basal sphenoid bone. With saggital cutting, the pituitary tumor was clearly distinguished from the hypothalamus, which was pushed out toward the ventriculus tertius.

The undifferentiated cells mainly occupying the tumor tissues were constructed of small chromophobe cells possessing poor and lightly stained cytoplasm (Figs. 1, 2). Tumor tissues mainly originated from the pars distalis of the hypophysis; however, it was not clear whether the tumorigenesis was expanded to the pars tuberalis. Tumor cells also invaded and proliferated in the ventriculus tertius and lateralis. Many irregular lobules surrounded by connective tissues were present throughout the pituitary tissues. Hemorrhagenous tissues composed of variously-sized follicle-like structures were observed. In the centers of tumorigenized lobules, many necrotic cells were observed. Twenty to thirty tumor cells were distributed in circles, the centers of which were filled with lightly eosinophilic fibrous structures (probably originating from neuron fibers). In addition to the blood vessels composed only of flattened endothelial cells, small vessels having thick walls, like sheathed arteries in the spleen, were observed. Many erythrocytes were fixed in these walls. Mitotic and gigantic figures of tumor cells were observed throughout the hypophysis.

Tumor tissues were clearly separated from the hypothalamus; however, bundles of neuron fibers continuing from the hypothalamus were observed to invade some tumor tissues. The pars intermedia and posterior were not tumorigenized anywhere. In the cavum hypophysis between the pars distalis and intermedia, proliferating cells were also observed. Some differentiated endocrine cells which possessed round and regular-sized nuclei were stained lightly by hematoxylin, and rich cytoplasm was observed to occupy the caudal small region of tumor tissue; however, their stainability by eosin solution was less than in control pituitary tissues.

Immunohistochemically, SV40 was expressed in the cell nuclei in the undifferentiated cell area, whereas the cells expressing several hormones were mainly distributed in the differentiated cell area (Figs. 3, 4). These gross anatomical and light microscopical results corresponded with those in a previous report (Maki et al., 1994).

Electron microscopically, the undifferentiated cell area was constructed of small cells mainly having nuclear diameters of 6-12 μm, and sometimes of giant cells with nuclei larger than 15 μm in diameter (Fig. 5). These small cells were divided into 2 types; electron-dense (ED) cells containing a little cytoplasm, and electron-lucent (EL) cells possessing relatively developed cytoplasm (Fig. 6). The nucleus was observed to have an obscured nucleolus and to show many notable invaginations of the nuclear membrane, to which most of the dense chromatin adhered. No intracellular micro-filamentous structures were observed. Many short cytoplasmic processes were irregularly elongated, and touched neighboring cells. No basement membrane was observed, while sometimes cytoplasmic processes were connected via gap junctions (Fig. 7). Erythrocytes and degenerated lymphocytes were distributed in the
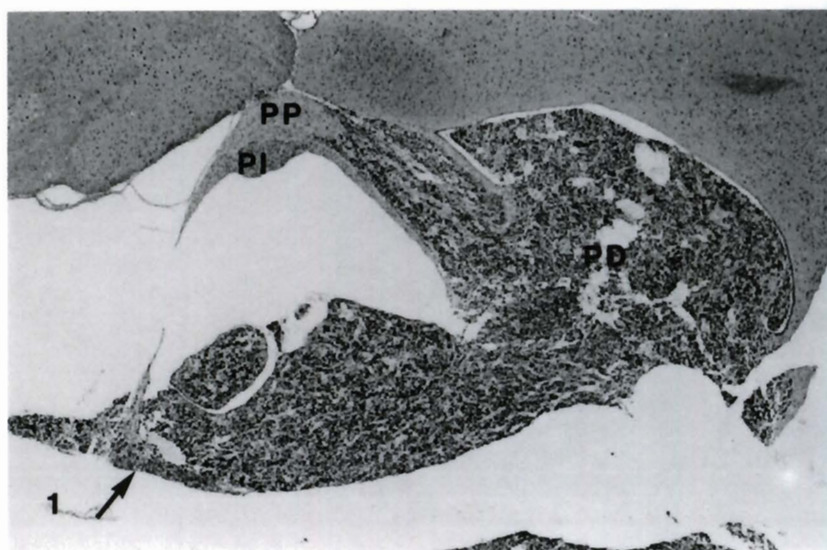

Fig. 1. Tumorigenized pituitary gland. Endocrine cells of the pars distalis (PD) are replaced by tumor cells which mainly have small nuclei. The pars intermedia (PI) and posterior (PP) are apparently normal. A differentiated cell area is observed in the caudal region of tumor tissues (arrow). Hematoxylin-eosin staining. x 50

intercellular spaces derived from hemorrhage. ED cells were composed of a poorly developed Golgi apparatus and rough endoplasmic reticulum, and a few small, round mitochondria. EL cells were composed of relatively developed intracellular organellas and elongated mitochondria. Many vacuoles were observed
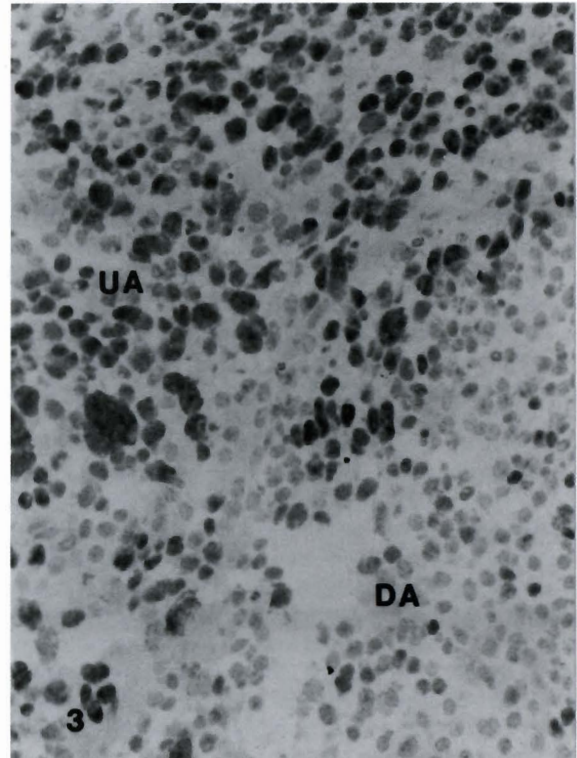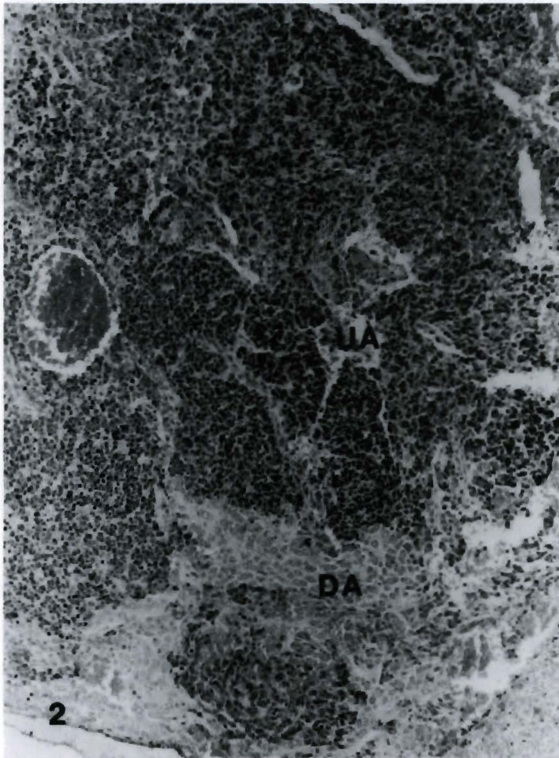

Fig. 2. Undifferentiated (UA) and differentiated cell areas (DA) in a pituitary tumor. They are diffusely adjacent without any boundary structures. Hematoxylin-eosin staining. x 100

Fig. 3. Distribution of SV40 large T antigen-positive cells. They are detected in the undifferentiated cell nuclei. UA: undifferentiated cell area; DA: differentiated cell area. Immunohistochemistry and hematoxylin counterstaining. x 350
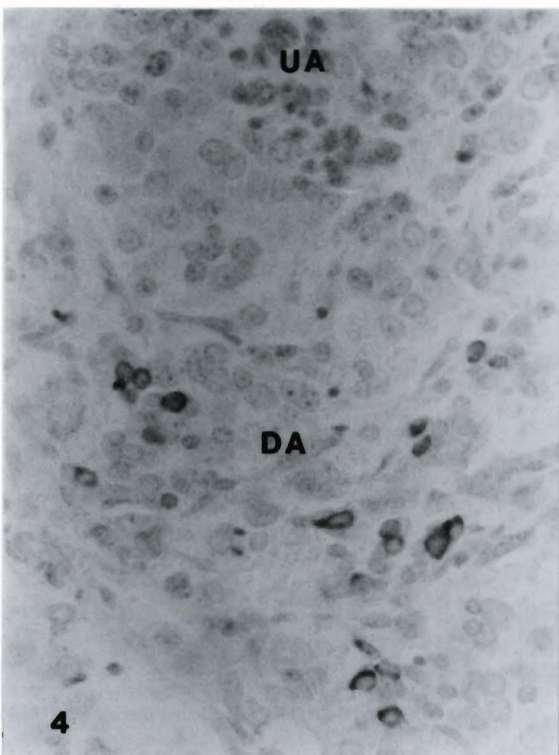
Fig. 4. Cells immunoreactive for GH in tumor tissues. They are distributed in the differentiated cell area (DA). Other cells immunostained with anti-hormone antisera are not shown. UA: undifferentiated cell area. Immunohistochemistry and hematoxylin counterstaining. x 430
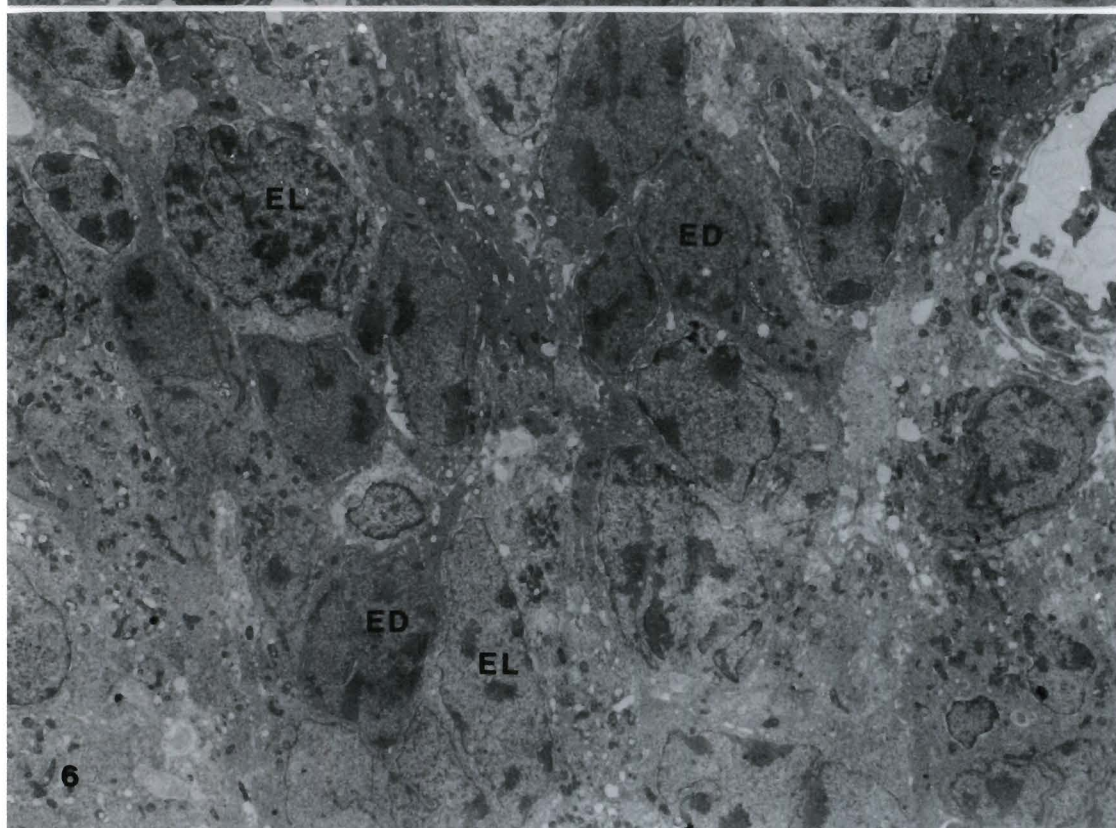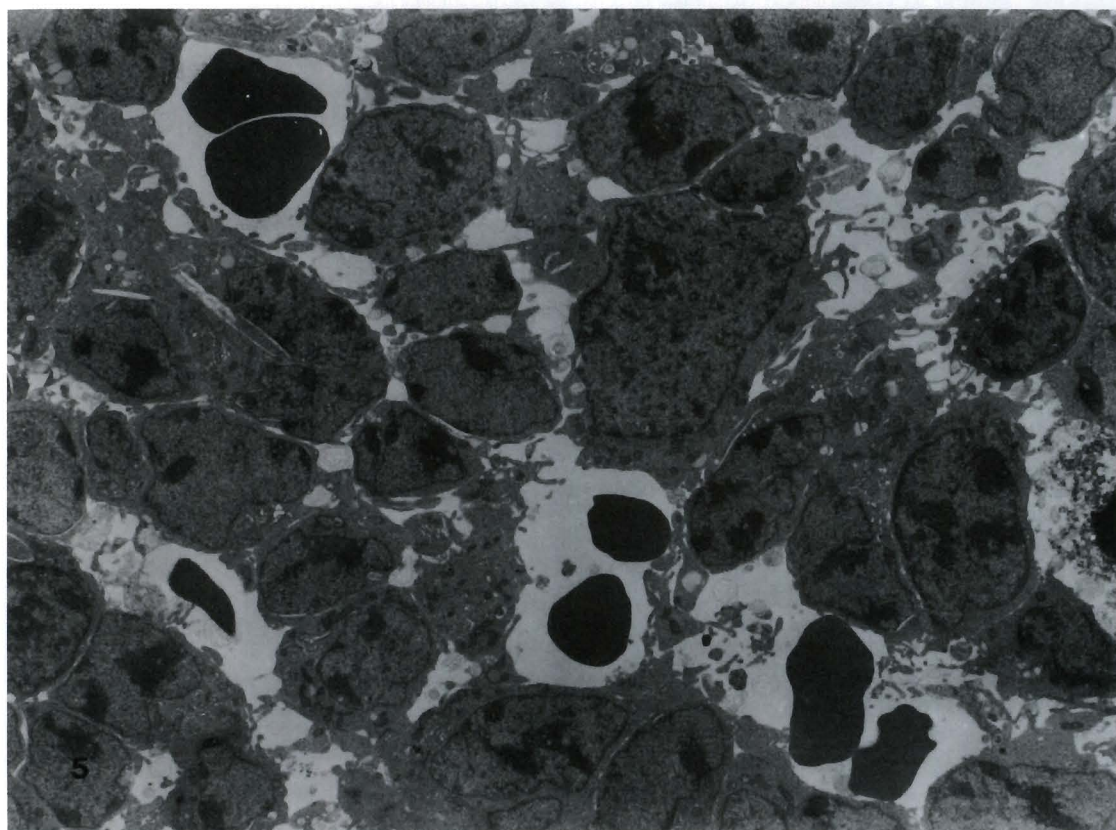
Fig. 5. Electron micrograph of the undifferentiated cell area. Small cells possessing many cytoplasmic processess are observed. Erythrocytes derived from hemorrhage are distributed in the intercellular spaces. x 3,100

Fig. 6. Electron micrograph of the undifferentiated cell area. Tumor cells in the undifferentiated cell area are divided into electron-dense (ED) cells and electron-lucent (EL) cells. x 3,100
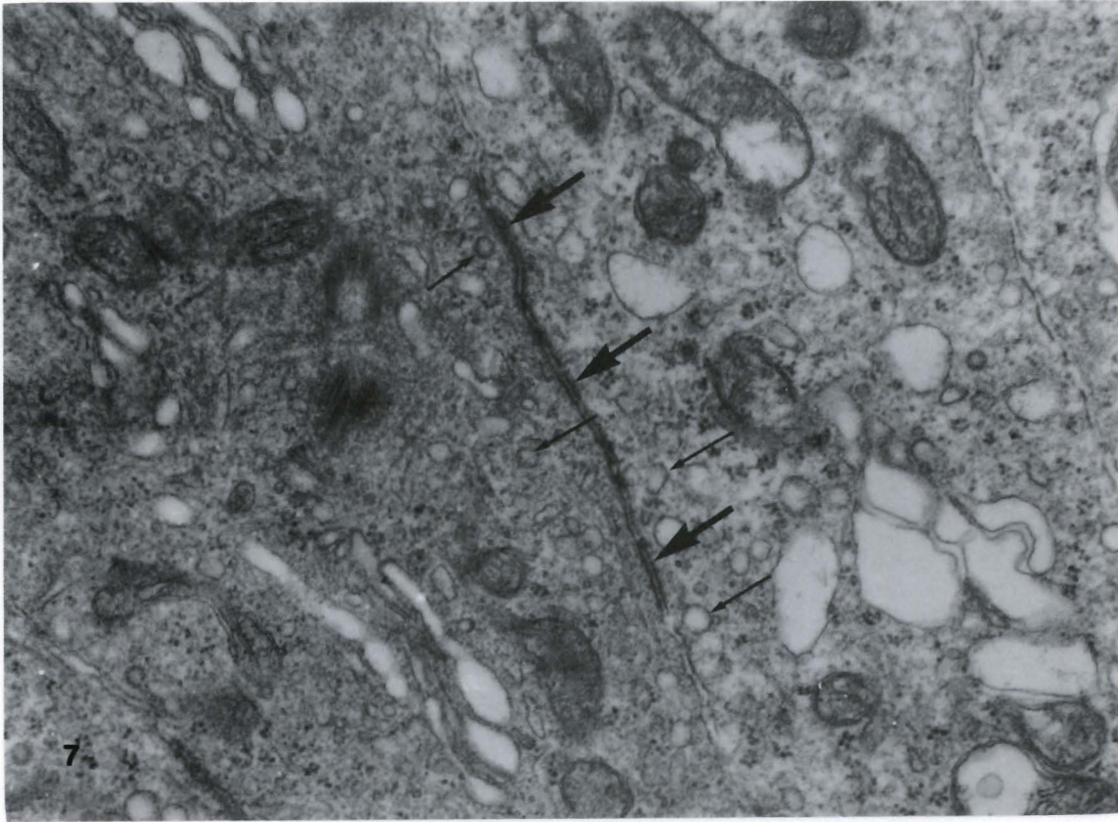
Fig. 7. High magnification of tumor cells. Gap junctions are observed between the cell membranes (large arrows). Many vacuoles (small arrows) are distributed below the cell membrane. x 3,100
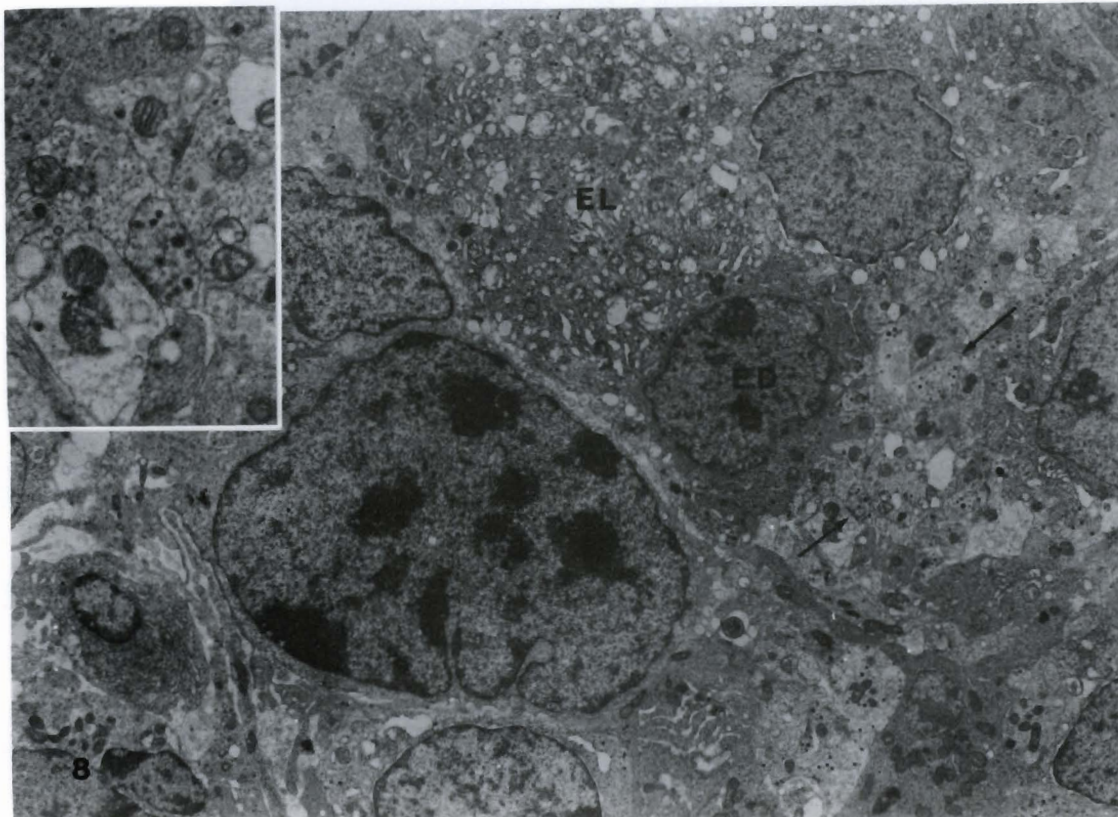
Fig. 8. Neuron fibers and synapse-like structures in the intercellular spaces of the undifferentiated cell area (arrows). Rarely, electron-lucent (EL) cells having abundant cytoplasm contain a few granules measured at about 80 nm in diameter. ED: electron-dense cell. x 4,800. An inset figure shows synapse-like structure. x 13,000
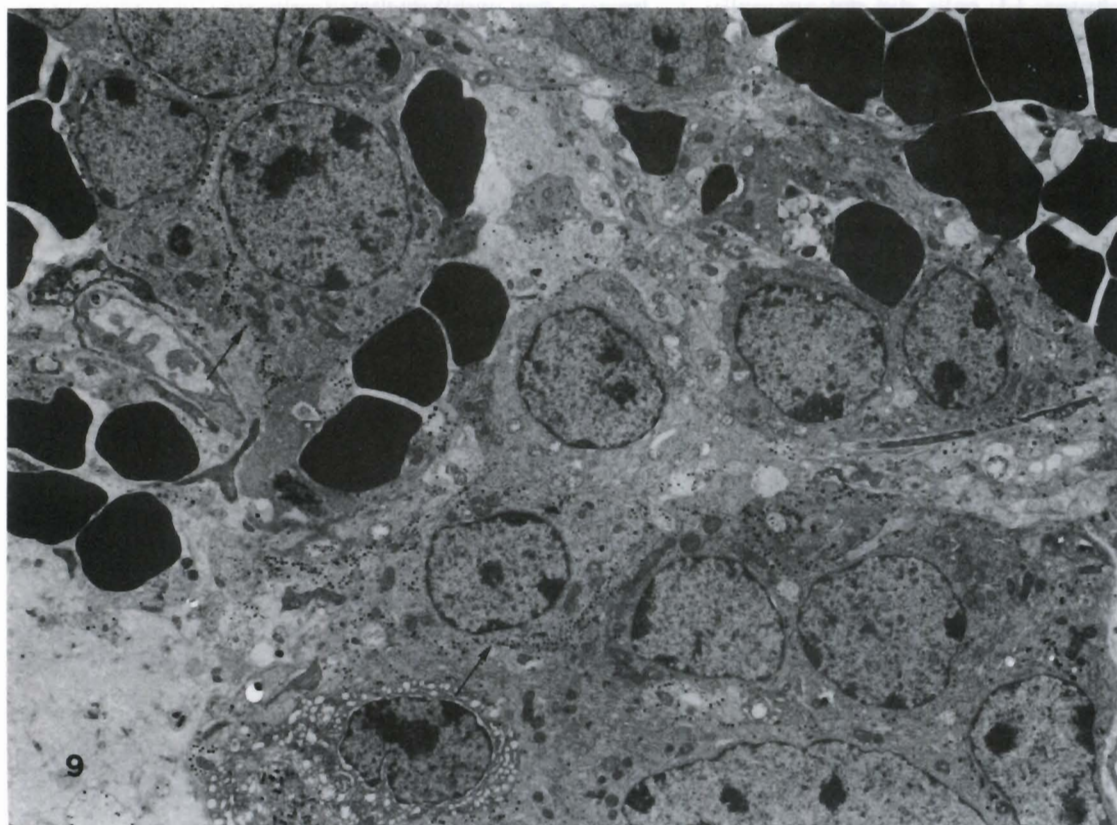
Fig. 9. Electron micrograph of the differentiated cell area. Endocrine cells possessing small granules which are distributed just below the cell membrane are observed (arrows). x 3,100
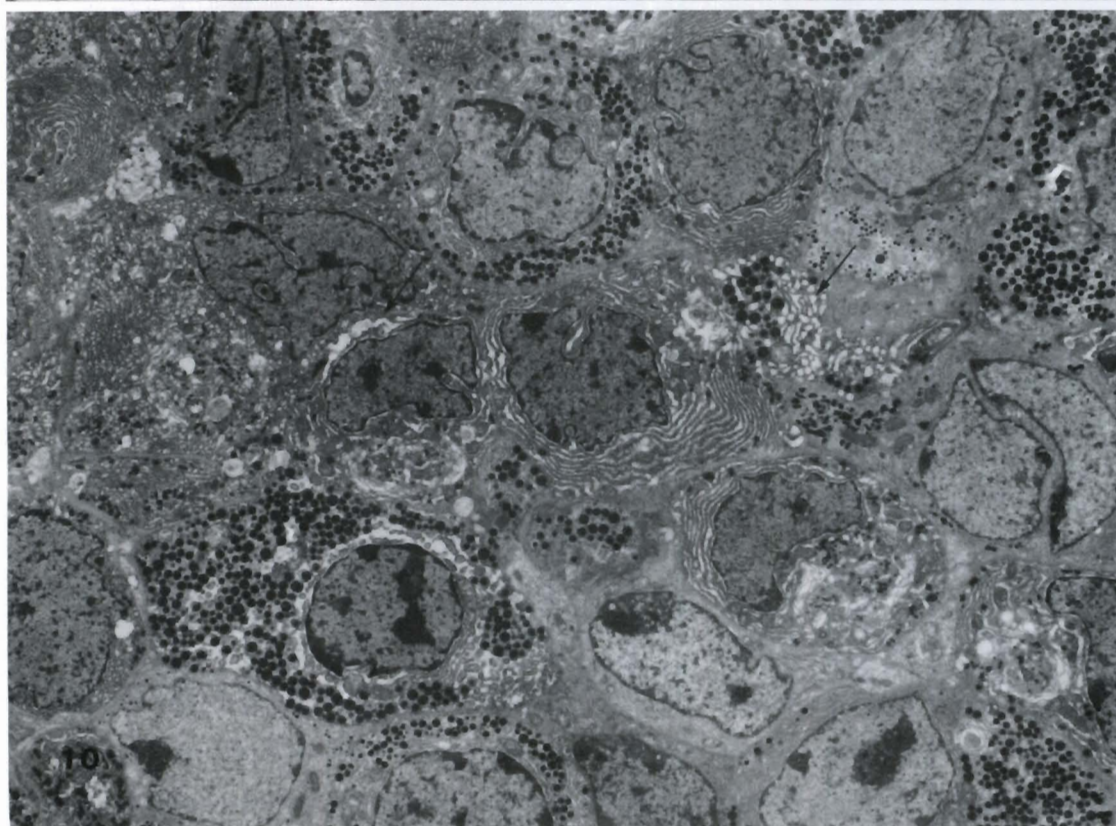
Fig. 10. Control section of the pars distalis in the pituitary gland. Variously-sized, electron dense secretory granules are observed. In some cells, the cisternae of the endoplasmic reticulum are dilated (arrows). x 3,100

below the cell membrane. EL cells did not generally possess as much granular material as ED cells. Rarely, a few dense granules measuring 80 nm or less in diameter and amorphous lysozomal granules were contained in the cells (Fig. 8). Sometimes, in the intercellular spaces, there were neuron fibrous and synapse-like structures. In the synapse-like structures, many electron-dense granules were observed. In the pituitary tissues of intact control animals, no undifferentiated cell area was demonstrated.

In the differentiated cell area, about 20 or more cell clusters were surrounded by a basement membrane (Fig. 9). The cells not showing any nuclear invaginations had nuclei measuring 7-10 μm in diameter. Rarely, large cells with nuclei over 15 μm in diameter were observed. The cell membranes directly contacting other cells were relatively smooth, and many gap junctions were detected. In most of these cells, many secretory granules were contained in the developed cytoplasm. Secretory granules, which were round and less than 100 nm in diameter, were more electron dense in smaller cells than in larger ones. They were distributed just below the cell membrane. The Golgi apparatus was well developed and the rough endoplasmic reticulum was dilated. Some-

times, a few undifferentiated cells similar to those in the undifferentiated cell area were distributed among these differentiated cells. In the control pituitary tissues, most of the granules of endocrine cells were over 100 nm in diameter, and mainly filling in the cytoplasm at about 350 nm. These granules were round and electron dense (Fig. 10).

Immunoelectron microscopically, positive reactions for SV40 large T antigen were observed in the nuclei in the undifferentiated cell area (Fig. 11). No polarity was detected by immunostaining. In the differentiated cell area, most secretory granules were labeled by GH, whereas only a small number of TSH-positive granules were detected, but their morphologies were not distinguishable from those in GH (Fig. 12).

Discussion

One hypothesis for the failure to produce TSH in the tumorigenesis of the TTP-1 transgenic mice is that the transferred gene binds to and interrupts the normal differentiation of pituitary hormones at an early stage of embryogenesis. Generally, it is unclear where the injected gene is transferred to. Thyrotroph embryonic
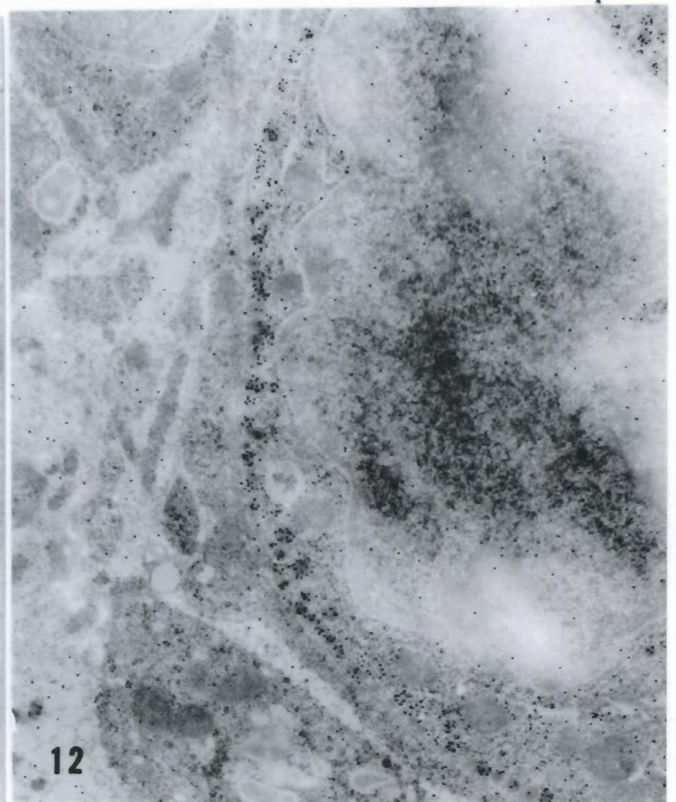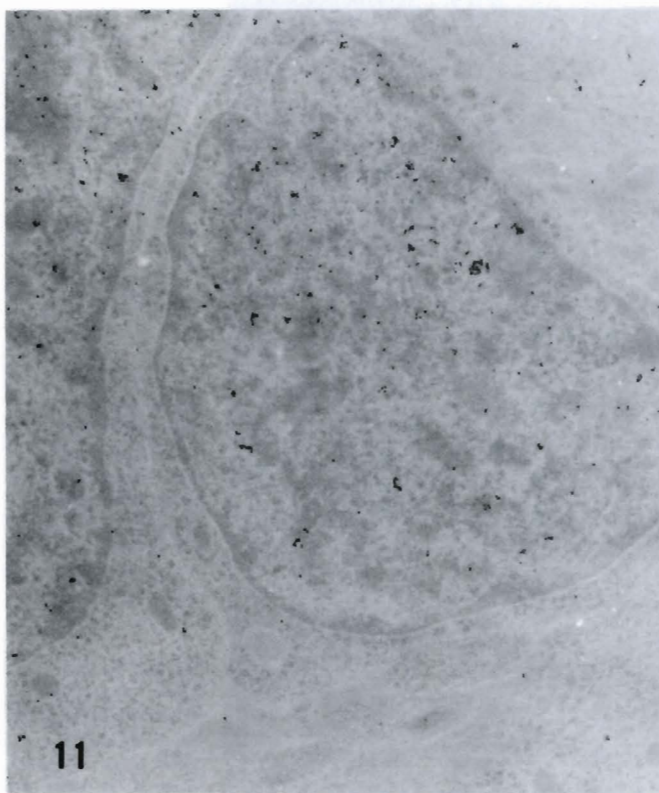

Fig. 11. Immunoelectron microscopy for SV40 large T antigen in the differentiated cell area. Gold particles are restricted to the nuclei. x 15,000

Fig. 12. Immunoelectron microscopy for GH in the differentiated cell area. Many small granules located just below the cell membrane are GH-positive. x 16,000

factor (TEF) and Pit-1, as pituitary-specific transcription factors, have been reported to act in the beginning of expression of TSH, PRL and GH genes in the anterior pituitary during embryogenesis (Bodner et al., 1988; Ingraham et al., 1988, 1990; Drolet et al., 1991). We speculate that the transferred gene first binds to TEF gene or Pit-1 gene and then begins to develop tumors in the progenitor cells of thyrotrophs during embryogenesis. In the present study, the undifferentiated cell area was diffusely adjacent to the differentiated cell area without any boundary structures. These results and clinical symptoms suggest that this tumor was malignant and of the progressing type. The differentiated cell area located in the caudal region of the hypophysis may have been composed of cells that resisted oncogenic tranformation or escaped against the effect of transferred gene (Maki et al., 1994).

Classically, pituitary adenoma, typical hormone secreting tumors have been divided by their cytoplasmic stainabilities into acidophilic adenoma, basophil adenoma and chromophobe adenoma. However, recent studies have suggested that this generally accepted classification is complicated and of limited value for pathological diagnosis (Kavacs and Horvath, 1986). Progress in biological methodology, for example, the introduction of immunohistochemical, electron microscopical, immunoelectron microscopical and in situ hybridization techniques, has contributed new information on the direct histopathological approach for detection of hormones from pituitary adenomas (Yamada et al., 1993). In the present and previous studies on classification of pituitary tumors generated in TTP-1 transgenic mice, targeted tissues were clearly defined as null cell (undifferentiated cell) adenoma/adenocarcinoma by immunohistochemical and immunoelectron microscopical techniques. Generally, cells expressing a certain mRNA have been reported to be identified with cells secreting the protein. The use of serial sections for immunohistochemistry and hybridohistochemistry reveals, however, more important information (Lloyd et al., 1992; Bachmann et al., 1993). For example, if the immuno/hybridohistochemistry result is -/+ in a certain cell, then the cell can produce the protein but not store it in its cytoplasm. Additionally, if the immuno/hybridohistochemistry is +/-, circulating proteins from the blood or intercellular spaces can be taken up by the cell (Kon et al., 1993). In the present study using electron microscopy, GH immunoreactive granules were detected in cells in the differentiated cell area; however, the Golgi apparatus and rough endoplasmic reticulum did not develop. This may suggest that production of pituitary hormones is stopped or very weak at the sampling period. Further studies for detection of mRNA using hybridohistochemistry are required.

In normal adenohypophysis, the diameters of secretory granules are generally measured at 200-350 nm in GH, 300-700 nm in PRL, 100-150 nm in TSH, 150-200 nm in ACTH and 200-250 nm in LH (Kurosumi, 1968). In the present study of so-called differentiated cell area, secretory granules less than 100 nm in diameter were positive for GH. The existence of such small granules has been reported in a previous study (Schechter et al., 1992) in which small secretory granules (80-100 nm) were found to be distributed in the tumorigenized pituitary cells of transgenic mice carrying a hybrid transgene composed of the 5'-flanking region of the human glycoprotein hormone α-subunit gene linked to the coding region of the SV40 large T antigen gene. In our unpublished data, undifferentiated area expressing SV40 large T antigen occupied small regions of pituitary gland at day 1 of birth, and then they grew day by the day. These findings suggest that the differentiated granulated cells do not receive sufficiently the effect of transferred SV40 gene. Further studies are required to confirm whether the granulated cells presented to differentiated cell area also express SV40 large T antigen using double staining method. Additionally, the fact that few granules were produced by the poorly developed Golgi apparatus, reminds us that the production system for secretory granules did not function at the sampling period.

If the tumor cells examined in this study were derived from fibroblasts or other connective tissues, many microfilamentous structures should have been detected; however, we were unable to observe these structures throughout the tumor cells. The existence of gap junctions among the tumor cells and of cells with relatively well-developed intracellular organellae, indicated clearly that the tumor cells examined in this study were derived from parenchymal cells.

In the present electron microscopical study, neuron fibrous and synapse-like structures were observed at the undifferentiated cell area. It is known that cytoplasmic processes of neurosecretory cells situated in the nucleus arcuatus, normally end at the pars tuberalis. The hypothesis that a part of the hypothalamus or neurohypophysis was surrounded by tumor tissues during embryogenesis was refuted by the fact that tumor tissues were clearly bounded by the above two tissues. These findings may suggest another possibility: that the tumorigenesis observed in the present study originated from the pars tuberalis and that it expanded to the pars distalis day by day after birth. However, in order to clarify the nervous characteristics, it is need to be specified in term of origin and neuropeptide content. In recent years, it has been suggested that the adenohypophysis is a kind of neuronal tissue with a neuroectodermal origin (Couly and Le Dourain, 1985). Some reports have also suggested that the folliculo-stellate cells of the anterior pituitary gland are a type of neural element (Cocchia and Miani, 1980; Nakajima et al., 1980; Velasco et al., 1982). It is possible that folliculo-stellate cells normally situated at the pars distalis are transformed into neuroendocrine cells by the carried oncogene. These hypotheses should lead to further ontogenetic investigations for studying tumor origin.

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