Invasive Pituitary Tumors in Female F344 Rats Induced by Estradiol Dipropionate*

HIROSHI SATOH,1 TETSUYO KAJIMURA,1 CHUN-JEN CHEN,2 KAZUTAKA YAMADA,3 KAZUHISA FURUHAMA,3 AND MAMORU NOMURA1

1Drug Safety Research Laboratory, 2Experimental Technology Research Center, and 3New Product Research Laboratories III, Daiichi Pharmaceutical Co., Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134, Japan

ABSTRACT

To clarify the histopathological progression of invasive tumors in the pituitary pars distalis due to estrogen, female Fischer 344 (F344) rats were treated subcutaneously with 5 mg/animal of estradiol dipropionate (ED) once every 2 wk for 13 wk. The animals were killed serially at 2-wk intervals during the investigation. The pituitaries with surrounding tissues were examined light microscopically. At week 7, pituitary cells showed proliferation and atypia with formation of blood-filled spaces. Lesions with these characteristics were diagnosed as adenomas. At week 9 or later, neoplastic cells exhibited extensive proliferation and infiltration into the surrounding tissues, suggesting development of carcinoma. Both proliferating cell nuclear antigen (PCNA) and 5-bromo-2'-deoxyuridine (BrdU) labeling index, markers of cell proliferation, were significantly increased in animals with adenoma or carcinoma. To detect sequential changes in pituitary weight, its signal intensity was periodically monitored in identical rats by using magnetic resonance (MR) imaging. The estimated pituitary weights revealed by MR imaging were comparable to the tumor weights obtained from rats at scheduled sacrifices. These results indicate that ED possesses the potential to cause carcinoma in rat pituitary and MR imaging is an effective tool for estimating the pituitary weight.

Keywords. Cell proliferation; immunohistochemistry; magnetic resonance (MR) imaging

INTRODUCTION

Although most of the spontaneous pituitary tumors in rats are thought to be benign, malignant tumors are recognized with a low incidence (6, 7, 10, 15, 17, 18, 20). In rats, there is a dearth of information dealing with xenobiotic-induced pituitary carcinomas, including estrogen-induced tumors. To our knowledge, estrogen has never produced malignant tumors in this species. No differences in morphological features between spontaneous and estrogen-induced tumors have been observed (15, 17, 23, 34, 38). In most cases of spontaneous pituitary carcinoma, tumor cells are invasive into the brain (19, 22), but a recent report shows that they preferentially infiltrate the surrounding tissues such as the capsule, surrounding veins, peripheral nerve, and sphenoid bone rather than the brain (8). The incidence of spontaneous pituitary tumors increases when the pituitaries are examined histopathologically together with surrounding tissues (8). Thus, it may be necessary to assess both the pituitary and surrounding tissues (including the brain and sphenoid bone) for diagnosis of malignant tumor. The pituitary with surrounding tissues has rarely been examined from a histopathologic viewpoint in previous studies dealing with spontaneous and estrogen-induced pituitary tumors.

According to recent reports, magnetic resonance (MR) imaging enables the in vivo evaluation of sequential changes in intracranial tumors (32, 33, 35). Therefore, much more information can be obtained from a single rat by using MR imaging. MR imaging may be useful for estimating pituitary weights, which is a key diagnostic tool for pituitary tumors (2, 4, 30).

In the present investigation, oleaginous estradiol dipropionate (ED), an estrogen derivative, was administrated to female rats to determine whether pituitary carcinomas with local invasion are seen when examined histopathologically together with the surrounding tissues. Furthermore, MR imaging was used to monitor tumor size and to measure sequential changes in pituitary mass.

MATERIALS AND METHODS

Animals

Seven-wk-old female Fischer 344/DuCrj rats weighing 115.7 to 136.3 g (Charles River Japan Inc., Yokohama,

<table>
<thead>
<tr>
<th>Table I.—Distribution of rats in the experiment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sacrifice period (weeks after the first administration)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Histopathological examination</td>
</tr>
<tr>
<td>Number of untreated animals</td>
</tr>
<tr>
<td>Number of ED-treated animals*</td>
</tr>
<tr>
<td>BrdU immunohistochemical and</td>
</tr>
<tr>
<td>Pituitary weight examination</td>
</tr>
<tr>
<td>Number of untreated animals</td>
</tr>
<tr>
<td>Number of ED-treated animals*</td>
</tr>
<tr>
<td>MR imaging examination</td>
</tr>
<tr>
<td>Number of untreated animals</td>
</tr>
<tr>
<td>Number of ED-treated animals*</td>
</tr>
</tbody>
</table>

*BrdU, 5-bromo-2'-deoxyuridine; MR imaging, magnetic resonance imaging.

*ED was administered subcutaneously once every 2 wk to rats. The animals were killed 1 wk after the last ED treatment.
Japan) were used in the investigations. They were housed 2 animals per wire-mesh cage in an air-conditioned room (temperature, 23 ± 2°C; relative humidity, 55 ± 15%) with a 12-hr light/dark cycle. Basal diet (F-2, Funabashi Farm, Chiba, Japan) and tap water were available ad libitum.

Experimental Design

Oleaginous estradiol dipropionate (ED; Ovahormon Depot®, 5 mg ED/ampule, Teikoku Hormon Mfg. Co., Ltd., Tokyo, Japan) was administered subcutaneously at 5 mg/animal to groups of 2 to 16 animals (Table I) every 2 wk for 13 wk (a total of 7 times). The dosage level of ED utilized in the present study was selected on the basis of previous reports (4, 21, 23, 24). Untreated animals served as the control. The animals treated with ED were killed by exsanguination under ether anesthesia at weeks 1, 3, 5, 7, 9, 11 and 13 after the first administration. Control animals were killed at weeks 7 and 13. Immediately after necropsy, the pituitary was carefully removed together with other surrounding tissues, including the capsule, surrounding veins, peripheral nerve, meninges, and sphenoid bone. These tissues were fixed in 10% neutral buffered formalin and then decalcified with buffered 25% formic acid for 10 days. Other main organs were examined for gross abnormalities and fixed in 10% neutral buffered formalin. The pituitary and surrounding tissues were trimmed sagittally and transversely to assist in detection of cellular invasion into the surrounding tissues (Fig. 1). The brain, lung, liver, kidneys, mammary gland, thyroid glands, adrenal glands, and all gross lesions were also trimmed. Afterward, the specimens were embedded in paraffin wax, cut at 3 μm, and stained with H&E for histopathological examination.

Histopathological Diagnosis

Pituitary lesions were classified according to the following 4 categories based on morphological features (8, 15, 17, 22): hypertrophy (enlargement of the cell size without cell proliferation); hyperplasia (focal or diffuse increases in number of hypertrophic pituitary cells intermixing with a small number of normal cells, with minimal compression of the brain); adenoma (a well-delineated mass with cellular atypia and a vascular pattern characterized by severe angiectasis, desquamation of the endothelial cells, and formation of blood-filled spaces); or carcinoma (evident invasion of the neoplastic cells into the surrounding tissues).

Immunohistochemistry and Morphometric Analysis

Proliferating Cell Nuclear Antigen Immunohistochemistry. For immunohistochemical detection of proliferating cell nuclear antigen (PCNA), the tissue sections mentioned above were processed by a labeled streptavidin-biotin (LSAB) method (37). In brief, the paraffin sections were deparaffinized, treated sequentially with mouse anti-PCNA antibody (1:500, DAKO Japan Co., Ltd., Tokyo, Japan), and stained with an LSAB kit (DAKO Japan Co., Ltd., Tokyo, Japan). The sections were then counters- stained with hematoxylin. Negative controls were per-

Table II.—Histopathological incidence of the pituitary lesions in rats treated with estradiol dipropionate.

<table>
<thead>
<tr>
<th>Sacrifice period (weeks after the first administration)</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of ED-treated animals*</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>12</td>
<td>6</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>2*</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Adenoma</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* ED was administered subcutaneously once every 2 wk to rats. The animals were killed 1 wk after the last ED treatment.

* Number of animals with pituitary lesions.
formed by substituting phosphate buffered saline for the primary antibody.

*BrdU Immunohistochemistry.* At weeks 7 and 13, 5 treated animals and 5 untreated animals were injected intraperitoneally with 100 mg/kg of BrdU (Sigma Chemical Co., St. Louis, MO, USA) 1 hr prior to kill for analyzing cell proliferation (Table I). After sacrifice, the pituitary was carefully dissected, weighed, and subjected to preparation of paraffin sections above. Proliferating cells were stained by use of an ABC-PO kit (Vector Laboratories Inc., Burlingame, CA, USA) with anti-BrdU antibody (1:100, Becton Immunocytometry System, San Jose, CA, USA). The peroxidase binding site was confirmed by the deposition of brownish dots.

*Morphometric Analysis.* In the PCNA or BrdU immunohistochemistry, the pituitary section was scanned by a microscopic color-image processor (SHR-6500, Keyence Co., Tokyo, Japan). The labeling index is the percentage of immunologically stained nuclei of the pars distalis.
Estimation of Pituitary Weight by MR Imaging

In a separate experiment, 5 animals treated with ED 7 times during the 13-wk period were imaged using a 4.7 T super conductive magnet MR imaging unit (Biospec CSI 47/40, Bruker Japan, Tokyo, Japan) at weeks 1, 3, 5, 7, 9, 11 and 13. Five untreated animals were imaged at weeks 7 and 13.

The animals were anesthetized by inhalation with 2.0% halothane (Takeda Chemical Industries, Ltd., Osaka, Japan) and placed in the supine position inside the radio frequent coil. T1 weighted image was periodically scanned by spin echo (recovery time, 1,000 msec, echo time, 15 msec; field of view, 600 mm) pulse sequence. An estimate of pituitary weight was obtained from the sagittal midline slice section in T1 weighted image according to the following formula (5):

\[ \text{estimated pituitary weight (mg)} = (\text{short diameter})^2 \times \frac{\text{longitudinal diameter}}{2}. \]
The estimated pituitary weight determined by MR imaging was then compared with the measured weight at scheduled sacrifices (weeks 7 and 13).

**Statistical Analysis**

Quantitative data were expressed as the mean value ± standard deviation (SD). Both pituitary weights and labeling indices were analyzed by Student’s t-test or Aspin–Welch’s test. In addition, the means of estimated pituitary weight by MR image and the actual pituitary weight in rats sacrificed on schedule were analyzed by Student’s t-test. A p value < 0.05 was considered significant.

**RESULTS**

**Histopathology**

The incidence of various pituitary lesions is summarized in Table II. In the control group, no remarkable changes were seen in the pituitary throughout the experimental period (Fig. 2a). In the ED-treated group, dilated Rathke’s cleft was first detected at week 1 (Fig. 2b), and the pituitary cells in the pars distalis showed diffuse hypertrophy with slightly dilated sinusoids at week 3. However, no atypical cellular features were detected. At week 5, a single adenoma was observed. An additional animal exhibited cellular hypertrophy and slight sinusoidal dilation. All animals at week 7 showed atypical neoplastic cells, which compressed the base of the brain, and small blood-filled spaces were observed in the pars distalis (Fig. 2c). At this time, 1 carcinoma showed invasion of neoplastic cells into the surrounding veins. After week 9, pituitary tumors showed severe angiectasis and hemorrhage into Rathke’s cleft (Fig. 2d, e). Furthermore, neoplastic cells infiltrated into the capsule (Fig. 3a), surrounding veins (Fig. 3b), meninges (Fig. 3c), or peripheral nerve (Fig. 3d). In addition, at week 13, 1 carcinoma exhibited neoplastic cell infiltration of the sphenoid bone marrow (Fig. 3e, f). The incidence of neoplastic cell infiltration in the capsule, surrounding veins, peripheral nerve, meninges, and sphenoid bone marrow was 96.3%, 55.6%, 22.2%, 14.8%, and 3.7%, respectively. There were no metastases of pituitary carcinomas to the other organs in this study.

**PCNA and BrdU Labeling Indices**

The PCNA labeling index in the ED treatment group was significantly increased in both adenomas and carcinomas, yielding indices of 1.78 and 3.16, respectively. Although the labeling index for hyperplasia (0.52) tended to be increased, there was not a significant difference from the untreated group (0.21).

The BrdU labeling index in the ED treatment group was significantly elevated for carcinomas (3.04) but not for adenomas (0.84) as compared to the untreated group (0.42) (Table III).

**MR Imaging**

In T1 weighted sagittal images, the control rat pituitary appeared triangular and hyperintense compared with the surrounding brain (Fig. 4a). At week 7 and later, the pituitary showed overt tumor formation with compression of the surrounding brain (Fig. 4b). At week 9 and later, the pituitary was markedly enlarged and showed variable intensity with respect to the surrounding brain (Fig. 4c).

**Estimated Pituitary Weight Determined by MR Imaging**

Actual pituitary weights and those estimated by MR imaging unit were significantly higher in ED-treated than in untreated groups (Fig. 5). MR imaging showed time-dependent pituitary enlargement in the ED treatment group and estimated pituitary weights of 49 ± 14 and 260 ± 12 mg at weeks 7 and 13, respectively (Fig. 5a). Meanwhile, the actual pituitary tumors weighed by balance in the ED treatment group at weeks 7 and 13 were 59 ± 11 and 244 ± 47 mg, respectively (Fig. 5b). When the estimated pituitary weight revealed by MR imaging was compared with the tumor weights obtained from rats at scheduled sacrifices, no statistical differences were determined.

**DISCUSSION**

Treatment of female rats with estradiol dipropionate (ED) induced pituitary carcinomas with local invasion. The earliest histologic change in the pituitary of rats treated with ED was dilation of Rathke’s cleft. At week 3, the pituitary showed cellular proliferation with hypertrophy and slight sinusoidal dilation. At week 5, 1 animal developed an adenoma, a finding that was earlier in onset compared to reports by others (23, 24, 33, 35). The border stage of hyperplasia and neoplasia was thought to be distinguished by a difference in cellular atypia and the occurrence of blood-filled spaces. These blood-filled spaces have been reported to be caused by local compression of either the sinusoid or superficial pituitary veins (19, 28, 35). At week 7, pituitary carcinoma was seen in 1 of 12 animals. This is the earliest reported ED-induced carcinoma in the rat. At week 9 or later, carcinomas were present in all ED-treated animals. The find-
FIG. 4.—T1 weighted sagittal section MR image of the pituitary. ×2. a) Untreated rat at week 13, displaying a normal pituitary (arrow). b) At week 7 of ED treatment, the pituitary (arrow) showed extensive growth and was more hyperintense than the surrounding brain. c) At week 13 of ED treatment, the enlarged pituitary (arrow) consisted of regions of hypo- and hyperintensity with marked compression of the overlying brain.

Findings indicate that adenomas progress to carcinomas between weeks 7 and 9. There has been some controversy regarding the differential diagnosis of pituitary carcinoma from adenoma, although local invasion or metastasis is recognized as definitive evidence of pituitary carcinoma (8, 15, 17, 22). In the present investigation, ED-induced carcinomas showed local invasion into the capsule, surrounding veins, peripheral nerve, meninges, or sphenoid bone marrow. Extracranial metastasis of pituitary carcinomas has not been observed in rodents, unlike cattle and humans (1, 16, 25, 27).

Estrogen-induced pituitary tumor in rats has been extensively explored as a model for prolactin-secreting tumors of human beings (9, 14, 21, 23, 24, 30–36, 38). However, these tumors are mostly adenomas with no local invasion. The discrepancy between our results and previous data (24, 31–36) may be largely due to sampling differences. In our study, removal of the pituitary together with the surrounding tissues (without dissecting them from the sphenoid bone) and controlled trimming allowed ready detection of local invasion. Since most investigators have prepared the tissue section only by routinely removing the gland from the sphenoid bone, local invasion may have been overlooked. In humans, cellular infiltration into the surrounding tissues is considered to occur with adenomas, but extensive local invasion or metastasis are indicators of malignancy (12, 13, 26, 29). This nomenclature is confused. These tumors are dis-

FIG. 5.—Comparison of actual and estimated pituitary weights. a) Estimated pituitary weight obtained from MR imaging. Estimated pituitary weight (mm³) = (short diameter)² × longitudinal diameter/2. Increases in estimated pituitary weight are found to occur in a time-dependent manner. b) Actual pituitary weight determined at necropsy. (open circles): untreated rats; (closed circles): ED-treated rats. ** p < 0.01 vs untreated controls. n = 5.
cussed by use of the term invasive adenoma which is believed to be aggressive neoplasm with little metastatic potential (3, 11). Consequently, ED-induced pituitary tumors with focal invasion may most closely resemble human invasive adenomas.

Although both PCNA and BrdU labeling indices were significantly increased with tumor progression, the former proved to be better than the latter for detection of cell proliferation in both adenoma and carcinoma. Hence, our data suggest that PCNA stain by a direct method using a paraffin section is superior to the indirect procedure with BrdU (preinjected) stain. Since animals developing pituitary tumors show a reduction in local blood flow (28, 32–34) as well as depression of the systemic circulation, the possibility exists that the uptake or transfer of BrdU into the pituitary is impeded by circulatory disturbances.

MR imaging was a useful in vivo method for sequential monitoring of pituitary tumors over time without a need to kill animals. In this study, however, MR imaging was not useful for detecting local invasion of pituitary carcinomas. Clifton and Meyer (2) and Fujimoto et al (4) have stated that the term pituitary tumor in rats is used to designate any pituitary gland weighing in excess of 30 mg. Our results demonstrated that all animals showed pituitary tumors by week 7 and pituitary weight was over 30 mg from this time on as estimated by MR imaging and weight at necropsy. In addition, there was no statistical difference between the pituitary weight revealed by MR imaging and the weight obtained from rats at scheduled sacrifice. In our careful histopathological approach, we described the importance of observing the pituitary together with the surrounding tissue. When the pituitary is removed from the sphenoid bone for weighing, the opportunity to evaluate this invasion is lost. In conclusion, invasive pituitary carcinomas were induced in rats treated with estrogen. Pituitary sections should be prepared with estrogen. Pituitary sections should be prepared with the surrounding tissues, including the sphenoid bone, to ensure an accurate diagnosis of pituitary neoplasms in histopathological studies. MR imaging is considered to offer the additional advantage of estimating pituitary weight without disruption of local architecture.

ACKNOWLEDGMENTS

The authors wish to thank Dr. F. Sekiguchi for his scientific advice and Mrs. Y. Ozaki, T. Hirota, and K. Aoyagi for their technical assistance.

REFERENCES


27. Yagi for their technical assistance.


STP HOME PAGE

Visit the World Wide Web Home Page for the Society of Toxicologic Pathologists:

http://www.toxpath.org

Contents include: headline news; information about membership; a listing of upcoming meetings; and information about Toxicologic Pathology, the official publication of the Society; as well as other STP publications.