Strain Difference in Regulation of Pituitary Tumor Transforming Gene (PTTG) in Estrogen-induced Pituitary Tumorigenesis in Rats

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Recently a novel oncogene, *PTTG* (pituitary tumor transforming gene) was isolated from a rat pituitary tumor cell line whose expression is apparently correlated with pituitary tumorigenesis. In the rat, estradiol (E2) is known to induce anterior pituitary hyperplasia. The effects of E2, however, vary greatly among rat strains. Therefore we examined the expression of *PTTG* and its regulation by E2 in F344, Wistar, Brown-Norway and Donryu rats. Four-week-old females were ovarioctomized and a pellet containing 10 mg of E2 was given s.c. Total RNA was isolated from the pituitary gland and *PTTG* mRNA was measured with a competitive RT-PCR technique. The F344 strain was the most susceptible to E2 induction of pituitary tumorigenesis, followed by Wistar and Brown-Norway, while no increase in pituitary weight was noted in Donryu rats. *PTTG* mRNA in the gland was induced by E2 within 48–72 h in F344 and Wistar, but not in Brown-Norway or Donryu strains. These data suggest that *PTTG* expression may at least in part be responsible for strain differences in E2-induced pituitary tumorigenesis.

Key words: Pituitary tumorigenesis — *PTTG* — Estrogen regulation — Strain difference — Competitive RT-PCR

The oncogene *PTTG* (pituitary tumor transforming gene) was first isolated from rat GH (growth hormone) tumor cell lines by differential mRNA display.1) Induction of *PTTG* alone results in transformation in NIH 3T3 cells, which become tumorigenic in vivo. Human *PTTG* was found to be expressed at only low levels in normal pituitary tissues but highly in secreting and non-secreting pituitary tumors as well as in a variety of tumor cell lines, including lung carcinoma, melanoma, leukemia, lymphoma and HeLa examples.2–4) More recent investigations have revealed that the gene is identical to securin, a sister chromatid separation inhibitor, whose overexpression may have an impact on cancer development by increasing genetic instability.5) Chronic treatment of rats with estradiol (E2) is known to result in the development of anterior pituitary tumors, which are initially benign masses and hormone-dependent, but may subsequently become autonomous. A study with F344 rats showed that *PTTG* mRNA in the pituitary gland increases in this process and that E2 may regulate the expression.6) However, susceptibility to E2 induction of pituitary tumors is highly strain-dependent.7, 8) The F344 strain is the most widely studied and the most sensitive to estrogen. Other rat strains including ACI, Wistar-Furth and Copenhagen have been reported to display estrogen-dependent growth and tumor induction.9–11) On the other hand, the Brown-Norway, Holtzman and Sprague Dawley strains appear to be insensitive or resistant.7, 12) When susceptible strains were treated with E2, the size of the pituitary gland started to increase immediately and steadily expanded without any evident focal growth.9) The designation of tumor is given on the basis of the size of the gland and the abnormal appearance of the cells, which are highly correlated.13) Thus, the E2-induced weight increase in the gland well represents the E2-dependent tumorigenicity. The hypothalamus is not essential for the E2 stimulation of pituitary growth, based on experiments involving transplantation of the pituitary gland.14) Several investigations have indicated the contribution of multiple genetic loci to this susceptibility.7, 15) To examine whether expression of *PTTG* and its regulation by E2 might be involved in E2-induced pituitary tumorigenesis, strain differences were assessed in the present study.

MATERIALS AND METHODS

**Animals** Female F344, SD, Donryu and Brown-Norway rats were purchased at 4 weeks of age from Charles River Japan Co. (Kanagawa). They were maintained with free access to basal diet and tap water. All animals were surgically ovarioctomized upon receipt and implanted with pellets containing 10 mg of E2 subcutaneously as described previously.16) For the time-course study, F344 rats were treated with E2 for 4, 8 and 13 weeks in the long term experiment (5 animals, each point), and for 12, 24, 48 and 96 h for the short term (4 animals, each point). For the comparison study among four strains, they were treated with E2 for 72 h for the short term effects and for 4 weeks for the long term effect (5 animals, each strain and treat-
ment). Animals were sacrificed under ether anesthesia. The pituitary gland and the uterus of each rat were weighed and immediately frozen in liquid nitrogen and stored at −80°C. Blood samples were collected from the abdominal artery and separated sera were stored at −20°C until assayed. All experiments were conducted under the guidelines of ‘A Guide for the Care and Use of Laboratory Animals of Hiroshima University.’

**Total RNA extraction and reverse transcription** Total RNAs were prepared from pituitary tissues with Isogen (Wakojunyaku, Osaka), a premixed RNA isolation reagent based on the acid guanidium thiocyanate-phenol-chloroform extraction method, following the supplied protocol. Aliquots were treated with 1 U of RQ1 DNase (Promega, Madison, WI) in 20 μl buffer containing 10 U of RNase inhibitor, RNasin (Promega) and 1 μg of RNA was reverse-transcribed with 200 U of MMLV-RT (Life Technologies, Rockville, MD) and 50 ng of oligo-dT primer in 12 μl of buffer containing 1 mM dNTP, 100 mM Tris HCl (pH 8.3), 150 mM KCl, 6 mM MgCl₂, 60 mM dithiothreitol and 5 U/μl RNasin with incubation at 37°C for 60 min.

**PCR primers** tPTTG-f1 and tPTTG-r with the sequences 5′-ATGGCTACTCTGATCTTTGTGTTAC-3′ and 5′-ATATCTGCATCGTAAC (581–600) were employed for tPTTG detection by PCR with the expected amplified size of 600 bp. For construction of the competitor, rPTTG-f2 and rPTTG-f2f1 (rPTTG-f1 linking to rPTTG-f1) with the sequences of 5′-TATCTGCATCGTAAC (581–600) and 5′-ATGGCTACTCTGATCTTTGTGTTAC-3′ were used. Primer sequences and conditions for G3PDH (glycerol-3-phosphate dehydrogenase) were chosen according to Weir et al.\(^\text{17}\) (the expected size of the amplified fragment was 534 bp).

**Construction of competitor DNA fragments** PCR amplifications were carried out with 20 pmol each of primers in a total volume of 50 μl containing 1.25 U of Ex-Taq DNase polymerase (TaKaRa Shuzo Co., Otsu), 0.2 mM dNTP and the supplied buffer. Each amplification was performed with 30 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, ending with 72°C for 5 min. PCR products were purified with agarose gel electrophoresis followed by cleaning with a GFX Gel Band Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). First, a 600 bp fragment was amplified by PCR with tPTTG-f1 and tPTTG-r from rat pituitary tumor cDNA. The DNA sequence was confirmed with an ABI PRISM 310 capillary sequencer (Applied Biosystems, Foster City, CA). Using this fragment as a template, the next PCR amplification with primers tPTTG-f2 and tPTTG-r was performed and the resulting 401 bp PCR fragment was gel-purified. Then the third PCR amplification was carried out from the 401 bp fragment with tPTTG-f1f2 and tPTTG-r to obtain a 421 bp competitor DNA for tPTTG.

**Competitive PCR** Sample cDNA (equivalent to 0.2 μg of total RNA) and various amounts (0–15 fg) of competitor DNA were co-amplified by PCR with Ex-Taq using the tPTTG-f1 and tPTTG-r primer set. The amplification conditions were as described above in a final volume of 20 μl. Each PCR product was electrophoretically separated on a 1.5% agarose gel containing ethidium bromide at 0.2 μg/ml. The image was digitized with a video capturing device, PrintGraph (Atto Co., Tokyo) and intensities of the blots were quantified using Scion Image software (Scion Corp., Frederick, MD). The log ratio of the blot intensities of sample cDNA over the competitor in each lane was plotted against amounts of the competitor. Quantity of rPTTG mRNAs was determined where the ratio was equal to 1.\(^\text{18}\)

**Prolactin (PRL) radioimmunoassay** Serum PRL was measured by radioimmunoassay with NIADDK reagents following the recommended protocol, the details of which have been reported previously.\(^\text{19}\)

**E₂ radioimmunoassay** Serum E₂ was measured with a radioimmunoassay kit for rat serum E₂, purchased from Immunotech, Inc. (Marseille, Cedex, France).

**Statistical analysis** Statistical comparisons were made using Student’s t test.

**RESULTS**

**Increase in pituitary weight and PTTG mRNA expression due to E₂ in ovariectomized female F344 rats** Significant increases in pituitary weight were noted one week after the implantation of a high-dose E₂ pellet. The increase continued linearly for 13 weeks. RT-PCR detection of PTTG mRNA indicated elevated expression of the gene in the pituitary tumor induced by E₂ (Fig. 1).

**Strain differences in E₂-induced pituitary weight increase** Absolute weights of the pituitary gland varied among the four different strains. In 5-week-old control groups, the smallest size of 4.4±0.2 mg was noted in Brown-Norway rats, while the average size was 13.0 mg in the Wistar strain. Four-week treatment with E₂ increased pituitary weights in a strain-dependent manner (Table I). The pituitary glands of F344 rats showed the highest sensitivity to E₂ in terms of weight increase. On the other hand, no weight increase in the gland was noted in Donryu rats. There were no differences in serum E₂ levels among the four strains of estrogenized rats, while the hormone was undetectable in ovariectomized animals.

**PTTG mRNA levels in different strains of estrogenized rats** Data for PTTG mRNA levels quantified by competitive RT-PCR in ovariectomized and estrogenized rats of the four different strains are summarized in Table II. The PTTG levels in ovariectomized rats varied among the strains. When the rats were treated with E₂ for 4 weeks, the level was significantly increased only in F344 rats.

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**Table I.** Strain differences in E₂-induced pituitary weight increase

| Strain      | Average weight (mg) | Increase (mg) |
|-------------|---------------------|---------------|
| Brown-Norway| 4.4 ± 0.2           |               |
| Wistar      | 13.0                |               |
| F344        | 12.0                |               |
| Donryu      | 3.0                 |               |

**Table II.** PTTG mRNA levels in different strains of estrogenized rats

| Strain      | Level of PTTG mRNA (compare to control) |
|-------------|----------------------------------------|
| Brown-Norway| 1.0                                    |
| Wistar      | 2.0                                    |
| F344        | 3.0                                    |
| Donryu      | 1.5                                    |
Early increase in PTTG mRNA in the pituitary gland in estrogenized F344 rats

Semi-quantitative RT-PCR indicated PTTG mRNA to be significantly increased 48 h after implantation of a pellet containing 10 mg of E2 (Fig. 2). Measurement confirmed rapid increase in serum E2 despite the s.c. administration route as a pellet.

Early changes in uterus weights and serum PRL levels in response to E2

Significant increases in uterus weights were noted in estrogenized groups of all of the strains at 72 h after E2 administration. PRL levels, however, differed among strains, increasing sharply in the F344 and Wistar cases, but not significantly changing in Donryu rats (Table III).

Early changes in PTTG mRNA due to E2 in the four strains of rats

Data for PTTG mRNA levels in the different strains of rat treated with E2 in pellets for three days, quantified by the competitive RT-PCR technique, are sum-
Rat PTTG Regulation in Different Strains

**DISCUSSION**

In the rat, chronic treatment with E$_2$ is known to induce anterior pituitary hyperplasia and results in tumors which are initially benign and hormone-dependent, but may subsequently become autonomous. The effects of the hormone, however, vary greatly among rat strains.\(^7,8\) Our data clearly demonstrated PTTG in the pituitary gland to be regulated by E$_2$ differently in each strain, with possible involvement in the strain differences in E$_2$-induced tumorigenesis.

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**Table II. PTTG mRNA Levels in the Pituitary Gland in Different Strains of Rats Treated with E$_2$ for 4 Weeks**

| Strain          | PTTG mRNA (fg/mg total RNA) | Increase (fold) |
|-----------------|-----------------------------|-----------------|
| F344 Ov         | 6.5±0.5*                    | 3.7             |
| F344 Ov+E$_2$   | 24±1.1**                   |                 |
| Wistar Ov      | 1.8±0.5                     | 1.7             |
| Wistar Ov+E$_2$ | 3.0±3.2                     |                 |
| Brown-Norway Ov| 5.9±0.9                     | 1.1             |
| Brown-Norway Ov+E$_2$ | 6.5±0.6       |                 |
| Donryu Ov      | 2.7±0.9                     | 1.1             |
| Donryu Ov+E$_2$ | 3.0±1.0                     |                 |

* a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E$_2$ was given when they were 5 weeks old.  
  b) Mean±SEM (n=5).  
  ** Significant difference from the respective ovariectomized group value (P<0.01).

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**Table III. Body, Pituitary, Uterus Weights and Serum E$_2$ and PRL Levels in Different Strains of Rats Treated with E$_2$ for 72 h**

| Strain          | Body weight (g) | Pituitary weight (mg) | Uterus weight (mg) | Serum E$_2$ (ng/ml) | Serum PRL (ng/ml) |
|-----------------|-----------------|-----------------------|--------------------|---------------------|------------------|
| F344 Ov         | 129±4.2*        | 7.9±1.4               | 30±3.8             | n.d.               | 23±2.5           |
| F344 Ov+E$_2$   | 100±4.2**       | 8.7±1.6               | 394±99.0           | 885±351**          | 179±51.0*        |
| Wistar Ov      | 212±5.2         | 10.8±0.6              | 73±12.9            | n.d.               | 59±15.0          |
| Wistar Ov+E$_2$ | 145±9.3**       | 12.4±1.2              | 361±16.7**         | 634±11.1*          | 227±96.1*        |
| Brown-Norway Ov| 135±0.9         | 6.6±0.7               | 47±2.3             | n.d.               | 21±2.0           |
| Brown-Norway Ov+E$_2$ | 100±3.4**      | 5.8±0.3               | 617±209.0          | 1005±204**         | 90±21.5*         |
| Donryu Ov      | 187±1.7         | 8.9±0.3               | 81±3.5             | n.d.               | 33±3.5           |
| Donryu Ov+E$_2$ | 157±1.1**       | 10.4±0.6              | 1125±26.7**        | 576±106*           | 36±8.4           |

* a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E$_2$ was given when they were 5 weeks old.  
  b) Mean±SEM (n=5).  
  c) n.d.: not detected.  
  *, ** Significant difference from the respective ovariectomized group value (* P<0.05, ** P<0.01).
Table IV. Induction of PTTG mRNA in the Pituitary Glands of Different Strains of Rats Treated with E2 for 72 h

| Strain          | PTTG mRNA (fg/mg total RNA) | Increase (fold) |
|-----------------|-----------------------------|-----------------|
| F344 Ov         | 8.4±1.0                    |                 |
| F344 Ov+E2      | 24±4.2                     | 2.9             |
| Wistar Ov       | 3.6±1.0                    |                 |
| Wistar Ov+E2    | 12.8±2.2                   | 3.6             |
| Brown-Norway Ov| 11.8±1.8                   |                 |
| Brown-Norway Ov+E2 | 15.8±1.8              | 1.3             |
| Donryu Ov       | 5.4±1.8                    |                 |
| Donryu Ov+E2    | 6.0±2.0                    | 1.1             |

* a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E2 was given when they were 5 weeks old.
  b) Mean±SEM (n=5).
  * Significant difference from the respective ovariectomized group value (P<0.01).

PTTG was first isolated in rat growth hormone-secreting pituitary cell lines by the mRNA differential display method. Two pituitary tumor-specific mRNAs were found to be overexpressed; one was the insulin-induced growth response protein and the other was the new gene, PTTG, with a cDNA encoding a 199-amino-acid, 25-kDa protein. Northern analysis in normal adult rats indicated that only testis contained significant amounts of PTTG mRNA, which was a truncated mRNA of approximately 1 kb in contrast to the 1.3 kb mRNA expressed in pituitary tumor cells. When a fibroblast cell line, NIH3T3, was transfected with a PTTG expression vector, it exhibited colony formation, anchorage-independent growth in soft agar and development of tumors in nude mice. Subsequent investigations revealed the human homologue of PTTG to exist as a 202-amino-acid protein. Human PTTG was found to be highly expressed in leukemias, lung carcinomas, lymphomas and HeLa cell lines as well as in pituitary adenomas. The protein contains both proline and basic amino acid-rich domains potentially involved in cancer-related signal transduction, and has been reported to be identical to a vertebrate sister-chromatid separation inhibitor (serurin), whose increased expression may result in genetic instability with chromosomal gain or loss.

When pituitary tumors are induced by E2, clear transitions between hyperplasia, adenoma and carcinoma are not apparent despite differences in cellular atypia. The size of the pituitary gland steadily increases without any evident focal growth. It is typical for pituitary adenoma to be detected as a hemorrhagic tumor, an example being shown in Fig. 1. The designation of tumor is given on the basis of the size of the gland and the abnormal appearance of the cells. Since the two are highly correlated, E2-induced weight increase in the pituitary gland was taken to represent E2-responsive tumorigenicity in the present study. Satoh et al. demonstrated pituitary glands to develop adenomas in all F344 rats treated with E2 for only 7 weeks, and the adenomas progressed to carcinomas featuring local invasion or metastasis within 13 weeks after the start of hormone exposure.

Susceptibility to E2-induced pituitary tumor is highly strain-dependent. We used four different strains which are commercially available. F344 is known to be particularly susceptible, while Donryu rats are very resistant as far as one month of E2 treatment is concerned. Interestingly, the normal sizes of the pituitary also differ among strains, being considerably smaller in the Brown-Norway case. The determined basal levels of PTTG mRNA also varied among strains, although they did not correlate with the normal pituitary weights.

Heaney et al. reported that PTTG mRNA in the pituitary gland increases early after E2 administration in F344 rats, in accordance with our data. While a period of time necessary to induce PTTG was within 24–48 h here, being slightly longer than the 12–24 h of the previous report, the increase in PTTG was definitely prior to development of pituitary hyperplasia. Whatever the interval for initiating the transcription was, the gene might not be under direct control of E2. We compared PTTG induction by E2 after 72 h in the different strains, this time point being chosen on the basis of our time-course study, and demonstrated the initial rise to be dependent on strain and correlated with susceptibility to pituitary tumorigenesis. It is well known that E2 stimulates PRL synthesis predominantly through activation of transcription. Serum PRL levels became extremely high in both F344 and Wistar rats when they were treated with E2 for 72 h, while, interestingly, only a moderate increase was noted in the Brown-Norway strain and no induction in Donryu rats. However, the classical investigation by Wiklund and Gorski, who compared pituitary tumor susceptibility between F344 and Holtzman strains, indicated that the lack of tumor formation in the Holtzman rat pituitary is not due to a general unresponsiveness to E2. They found increased DNA synthesis during the first 2–4 days of E2 treatment and then a return to normal afterwards. On the other hand, elevation of PRL was seen as a primary response to E2, which was sustained on prolonged E2 treatment. This seems not to be the case in the Donryu strain which showed virtually no elevation in serum PRL level at 72 h after estrogenization in the present study. The comparison between ACI and SD rats by Stone et al. however, gave findings consistent with our results, PRL induction by E2 in ACI being much higher than that in SD rats on day 2 of diethylstilbestrol (DES) treatment. It should be pointed out that the so-called general responsiveness to E2 in the pituitary gland has not been quantitatively assessed in a thorough manner. Serum E2 levels were high in all four strains in the present
study, indicating no difference in the E₂ metabolism. The genomic structure of rat PTTG has been characterized and at least a 745 bp 5′ flanking sequence was revealed to be required for transcriptional activation. Transfection experiments with a rat PTTG-5′-flanking luciferase reporter in a rat pituitary cell line, GH3, showed that E₂ could significantly induce transcription through the promoter, albeit the effect was very weak. Since there is no evidence to indicate a direct relationship between promoter sequence and E₂ receptors, activation by E₂ may be indirect, although the response clearly requires E₂ receptors according to our recent results (unpublished). Although evidence for involvement of PTTG in pituitary tumorigenesis appears to be convincing, other genes may also contribute to strain differences. Ying found that p53 and rb mRNAs were increased in response to DES treatment in SD, but not in F344 rats. C-fos has also been reported to be regulated by E₂ in a specific manner in the pituitary gland of F344 rats.

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