Establishment of Estrogen Receptor-positive Transplantable Rat Thyroid Tumor Cell Lines in Vivo

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ABSTRACT

We established 17 transplantable rat thyroid tumor cell lines from the primary thyroid tumor of rats induced by N-bis(2-hydroxypropyl)nitrosamine. Among the 17 tumor cell lines established, only two of them (D1 and G1) were estrogen receptor (ER) positive. These two cell lines were characterized with respect to transplantability, histological features, ER contents and cellular localization, and expression of ER message. The ER contents, determined by dextran-coated charcoal assay, were 13.3 and 20.7 fmol/mg protein for D1 and G1 cell lines, respectively. Scatchard plot analysis indicates that the dissociation constants (K_d) were 0.17 and 0.4 nM, respectively, for D1 and G1 cell lines. Sucrose density centrifugation analysis detected a hormone-receptor complex which sedimented at the 4S region, characteristic for ER. Immunohistological staining revealed that the ER was localized in the nuclei. The presence of ER in D1 and G1 cell lines was further confirmed by reverse transcriptase-polymerase chain reaction to detect the ER mRNA. These results demonstrated that ER is expressed in some thyroid tumors. The ER-positive transplantable tumor cell lines are useful for studying the direct effect of estrogen on thyroid tumors in vitro and in vivo.

INTRODUCTION

It is well known that TSH^3 plays an important role in regulating the growth and biological functions of thyroid cells. Previously we reported that induction of rat thyroid tumors by DHPN can be enhanced by postiniatination treatment with 3-amino-1,2,4-triazole (1), phenobarbital (2), and propylthiouracil (3), all of which cause elevation of serum TSH levels. The incidence of human thyroid disorders, including cancer, in women is 4 times higher than that of men. The lesions in young women are usually well differentiated and have a good prognosis (4, 5). The effect of age and sex, however, on the incidence and prognosis of thyroid neoplasm cannot be explained by the increased level of serum TSH. Clinical data suggest that sex hormones, especially estrogen, may play a role in the development of thyroid neoplasm (6, 7). The presence of ER in the thyroid tissues may indicate potential sensitivities of the cells to estradiol. This hypothesis is supported in a variety of cancers including human breast cancer, testicular, pituitary, uterine, and renal tumors.

Recent reports have demonstrated the presence of ER in a variety of benign and malignant human thyroid tissues (8–18). Although the amount of ER in these tissues is low, relative to that in breast cancer, these results indicated that estrogen may exert its effect directly on thyroid tissues. The biological function of ER in the development of thyroid malignancy needs to be established. The present study establishes the estrogen receptor in positive, transplantable thyroid tumors, facilitating further studies on the relation between ER expression and tumour growth.

MATERIALS AND METHODS

Animals. Male Wistar rats were obtained from Shizuoka Experimental Animal Farm (Shizuoka, Japan).

Reagents. DHPN was purchased from Nakarai Chemical Co. (Kyoto, Japan); [3H]estradiol and 17ß-methyl-[3H]methyltrienolone from New England Nuclear; 17ß-estradiol and methyltrienolone from Sigma Chemical Co. (St. Louis, MO); ER-ELA monoclonal antibody kit from Abbott Laboratories (Chicago, IL); horseradish peroxidase goat anti-rat F(ab′)^2; fragment from Amersham Corp. (Braunswick, Germany); rabbit polyclonal anti-human thyroglobulin from Dako; avidin-biotin peroxidase complex kit from Vector Laboratories; avian myeloma virus-RT and Taq polymerase from Pharmacia LKB Biotechnology; RNAzol from Biotecx Laboratories; NuSieve from Toyobo (Japan).

Transplantable Rat Thyroid Tumor. The induction of rat primary thyroid tumor was described previously (1). Briefly, male Wistar rats were given three weekly i.p. injections of 210 mg/100 g body weight of DHPN and fed on basal diet for 30 weeks. The primary thyroid tumors generated by this experimental regimen were removed, minced under sterile conditions, and washed in 0.9% saline solution. Inbred Wistar rats (4 weeks old) were given s.c. implants with the same quantity of thyroid tissues (20 mg) under anaesthesia. All animals developed tumors at the inoculated sites; these tumors were designated as the first passage. Tumors of the second, third, and fourth passages were obtained by serial transplantation in newborn rats.

Morphological Observation. For light microscopic studies, primary and transplantable tumors were fixed in 10% formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin. For the localization of thyroglobulin, 4-μm-thick frozen sections were fixed in cold acetone (−20°C) for 10 min, and washed with two changes of PBS solution. Sections were incubated with anti-anti-thyroglobulin (1:1, 500 dilution) at room temperature for 1 h. Nonspecific background activity was suppressed using 1% horse serum. Sections were visualized by avidin-biotin-peroxidase complex method. Human thyroid tissue section served as a positive control.

DCC Assay for Estrogen Receptor. [(2,4,6,7,16,17-3H)Estradiol (141.9 Ci/mmol) was used as radioactive ligand. All specimens stored at −90°C were weighed; homogenized in cold 10 mM Tris-HC1 (pH 7.4), 1.5 mM EDTA, 0.5 mM dithiothreitol, and 10 mM sodium molydate buffer (pH 7.4); and centrifuged at 105,000 × g for 1 h. The resultant supernatant was used as the cytosolic fraction and its protein content was determined by the method of Lowry et al. (19). Cytosol (100 μl) was incubated with different concentrations (1–40 nM) radioactive estradiol in the presence and absence of a 100-fold excess of nonlabeled 17ß-estradiol for 16 h at 4°C. The DCC solution was then added to the mixture and incubation was continued for an additional 10 min. The samples were centrifuged at 3000 rpm at 4°C for 10 min. The radioactivity in the supernatants was determined by liquid scintillation counting. The quantitation of ER was carried out by Scatchard analysis (20) and by EIA using monoclonal antibody (H222). Androgen receptor assay was performed using the 3H-labeled synthetic analogue methyltrienolone ([3H]R1881).

Sucrose Density Gradient Analysis. To determine the size of estrogen receptor complex, sucrose density gradient analysis was performed (21). Aliquots (200 μl) of the reaction mixture from estrogen receptor assays were layered separately onto a 5–20% sucrose gradient in buffer containing 10 mM Tris-HC1 (pH 7.4) and 0.4 mM KCl and centrifuged in a SW 40 Ti rotor at 33,000 rpm for 16 h. Fluorescence-labeled bovine serum albumin was included as an internal size
standard. After centrifugation, a 150-μl fraction was collected and the radioactivity was determined by liquid scintillation counting.

**Immunohistochemical Localization of ER.** To determine the cellular localization of ER, a modified immunohistochemical technique of Yamashita et al. (22) was used. The primary antibody (H222) was a monoclonal antibody directed against ER protein of MCF-7 human breast cancer cells. HRP-labeled goat anti-rat F(ab′)₂ fragment, HRP-F(ab′)₂, was absorbed with rat normal serum to diminish nonspecific absorption. Frozen sections (4 μm) fixed with Zamboni’s solution were incubated with 5% skim milk solution overnight, followed by incubation with monoclonal antibody (H222) for 2 h at room temperature. After a washing with PBS, the sections were incubated for 30 min with anti-rat F(ab′)₂ fragment antibody conjugated with HRP. The sections were washed with PBS and incubated with 3,3′-diaminobenzidine containing 0.03% hydrogen peroxide solution for 5 min. The sections were counterstained with Harris hematoxylin solution. MCF-7 cultured cells were used as a positive control.

**Detection of Rat Estrogen Receptor mRNA.** Detection of estrogen receptor mRNA was by RT-PCR of total cellular RNA using rat ER-specific oligonucleotide primers spanning a 390-base pair fragment of the ER gene (23). Total cellular RNA from approximately 50 mg of tumor tissue was isolated by the RNAzol method (Tel-Test, Inc., Texas). RNA extracted from rat uterus served as a positive control.

RT-PCR method was performed as described previously (24). Synthesis of complementary DNA was carried out in a 10-μl reaction volume containing 1 μg of total RNA, 2 μM primer, and 5 units of avian myeloma virus-RT. After heat inactivation of the RT at 92°C, 2.5 units of Taq DNA polymerase was added to 50 μl of the PCR reaction mixture (K, 0.6—1.7—0.17—0.4

**RESULTS**

**Establishment of Transplantable Rat Thyroid Tumors.** s.c. inoculation of 19 primary tumors into inbred male rats yielded 17 transplantable rat thyroid tumor cell lines (Table 1). Macroscopically, most tumors grew in the s.c. tissue of inoculation sites. No tumor formation in other sites was observed. The histological classification of the tumors is summarized in Table 1. Most tumors were composed of follicles with little or no colloid and were lined by tall and cuboidal epithelial cells. The epithelial cells were characterized by hyperchromatic nuclei and basophilic cytoplasms, with partial papillary proliferation. Eight tumors were diagnosed as papillary carcinomas, five as follicular-papillary carcinomas, and four as follicular carcinomas. Immunohistochemical staining revealed that thyroglobulin was present in follicular epithelial cells (data not shown).

**Assay of Cytosol ER.** In the DCC ER assay, 2 of 17 transplantable tumor lines (D1 and G1) showed positive reactions, with the of 13.3 and 20.7 fmol/mg of protein, respectively (Table 1). Fig. 1 shows a representative result of Scatchard plot analysis. The Kₐ values obtained from this analysis were 0.17 and 0.4 nM, respectively, for D1 and G1 tumor lines (Table 1; Fig. 1). Furthermore, two tumor lines (A1—1 and C1) were androgen receptor positive; the receptor contents and binding affinities (Kₐ values) were 8 and 22 fmol/mg protein and 0.3 and 0.8 nM, respectively. However, none of these tumor lines concurrently expressed both ER and androgen receptor.

The ER contents in D1 and G1 tumor lines were further confirmed by a more sensitive EIA using ER-EIA kit (Abbott Laboratories). The ER contents thus obtained in D1 and G1 cells were 12.9 and 17.5 fmol/mg protein, respectively. The results from both assays were in agreement.

**Sucrose Density Gradient.** The size of the ER complex was determined by sucrose density gradient centrifugation analysis. In the presence of 0.4 M KCl, the complex sedimented at the 4S region (Fig. 2). In contrast, in the absence of KCl, the ER complex sedimented at two regions, 4S and 8S (data not shown).

**Immunohistochemical Localization of ER.** Immunohistochemical staining with a monoclonal antibody (H222) demonstrated that the specific staining for ER was located exclusively in the nuclei of the follicular epithelial cells in which thyroglobulin was detected (Fig. 3). No staining was observed in the cytoplasm and plasma membrane.

These results agreed with those determined by DCC assay (Table 1; Fig. 1).

**Detection of Rat Estrogen Receptor mRNA.** To ascertain whether the presence of ER in tumor cell lines correlates with the

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histology</th>
<th>ER content (fmol/mg protein)</th>
<th>Androgen receptor content (fmol/mg protein)</th>
<th>Affinity (Kₐ, 10⁻⁶ M)</th>
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<tr>
<td>A1-1</td>
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<td>Papillary, poorly</td>
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<td>A4-1</td>
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<ref>Abbott Laboratories</ref>
expression of ER mRNA, a RT-PCR technique was used. As shown in Fig. 4A, the ER message was found only in tumor cell lines (Lanes 2, 3, and 4) containing ER molecules as detected by the binding assay, but not in the tumor cell lines with no detectable ER molecules (Lane 5). The identity of the RT-PCR amplified product from transplantable tumor cell lines was determined by comparing the restriction enzyme digestion pattern of product amplified from rat uterus. PstI restriction enzyme digestion generated three fragments of the expected sizes (171, 112, and 107 base pairs) in both cases (Fig. 4B).

DISCUSSION

In this study, we developed 17 transplantable rat thyroid tumor cell lines from primary thyroid tumors of rats induced by DHPN, and demonstrated for the first time the presence of ER in two of the cell lines (D1 and G1). The majority of those cell lines were ER negative. The presence of ER in D1 and G1 cell lines was detected by the DCC method and analyzed by Scatchard plot to determine the quantity and binding affinity of ER, by immunohistochemical staining for the localization of ER molecules, and confirmed by RT-PCR technique to identify the ER message.

Previous studies utilizing ligand binding assays to assess the presence of ER have generated conflicting results (8–12, 14). Clark et al. (9) reported that neoplastic thyroid tissue had an approximately 4-fold higher ER level than nonneoplastic thyroid tissue removed from the same patients and that the dissociation constant (Kd) for ER did not differ in nonneoplastic and neoplastic cases. In contrast, Hample et al. (10) reported that the concentration of ER, in both goitrous and neoplastic thyroids, exhibited no significant difference. Interpretation of results obtained from ligand-binding assays were difficult in cases where receptor concentration was low or where only a specific cell type within a tissue contained ER. To circumvent this dilemma, we adapted a modified immunohistochemical method (22) using a monoclonal antibody with specificity to the ER protein to determine the cellular localization of the ER. Our results clearly indicated that ER was localized exclusively in the nuclei of the follicular epithelial cells but not in the cytoplasm or the plasma membrane, in agreement with that of previous reports (13, 17).

Increasing the level of detection of ER in a variety of carcinomas of the thyroid by immunohistochemical methodology has been recently documented (18). Despite the higher sensitivity of the immunohistochemical method over the biochemical assay (DCC method), the results from both techniques correlate well. Among 17 transplantable thyroid tumor cell lines established, only two of them were ER positive as determined by both methods.

The size of ER was evaluated by sucrose density gradient analysis. In the presence of 0.4 M KCl only 4S type of ER complex was detected. In contrast, both 4S and 8S types of ER complex were detected in the absence of salt. Similarly, both 4S and 8S types of ER complex were also found in breast carcinomas (12). Interestingly, the 4S complex was predominantly detected in the thyroid tumors as compared to normal thyroid tissue (8). Unfortunately, in those studies the salt concentration under which the 8S ER complex was detected was not specified. The generation of 8S ER complex could be due to the aggregation of the receptor molecules in the absence of high salt. We used a highly sensitive method (RT-PCR) to detect the expression of ER mRNA and unequivocally demonstrated that the ER message was detected only in tumor cell lines in which ER molecules were detected by other techniques.
Studies on the effect of female steroid hormones on human tumor cell growth are mainly focused on breast cancer. The growth of breast tumor cells is stimulated by estrogen but is inhibited by estrogen antagonists (e.g., tamoxifen) (25); these effects could be mediated through ER. The dependence of tumor growth on estrogen and other steroids is noted to have important pathophysiological and therapeutic implications in a number of malignancies including breast cancer (25-27), prostate cancer (28), and lymphatic leukemia and malignant lymphoma (29). The presence of ER, however, does not imply that ER has a biological function in the cells. It is suggested that some breast cancers show no response to estrogen antagonists in spite of the presence of ER in those tumors (25). Moreover, ER is known to be present in many other tumors of nontarget organs for estrogen, such as gastric carcinoma, melanoma, lung cancer, and renal cell carcinoma (30-33). The biological role of ER in these tumor cells is unknown. A recent study demonstrates that ovariectomy, but not castration, reduced the incidence of thyroid carcinoma in experimental animals (34). Furthermore, the progression of histological malignancy of thyroid tumors correlates positively with the ER contents (35). The biological evolution of thyroid tumors in relation to ER contents needs to be established. These data, taken together, indicate that the development of some thyroid neoplasms may be affected by estrogen. If the progression of thyroid tumors is dependent on estrogen, it is conceivable that endocrine therapy using antiestrogens may be an effective approach for the management of thyroid tumors.

It is well documented that estradiol treatment induces overexpression of cellular oncogenes (c-fos, c-myc, N-myc, ras, and c-erb-B) in the target tissues (36-39). The ER-positive transplantable thyroid tumor cell lines (D1 and G1) established in the present study are useful for studying the direct effect of estrogen on thyroid tumors in vitro and in vivo. This cell system may facilitate our understanding of the mechanisms underlying the development of thyroid tumors via the interaction of estrogen and its receptor and the subsequent effect on cellular oncogenes resulting from these interactions.

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REFERENCES


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