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Measurement of Estrogen and Progesterone Receptors in Abnormal Human Parathyroid Tissue

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Summary. Estrogen and/or progestin administration to postmenopausal women with primary hyperparathyroidism lowers serum calcium. We measured cytosolic estrogen receptors (ER) and progesterone receptors (PR) by classical hormone-receptor binding techniques in parathyroid tissue removed from 10 men and 20 women, and ER by immunocytochemistry in tissue from an additional one man and seven women in order to ascertain whether these agents might exert a direct effect upon tissue responsible for hyperparathyroidism. ER were negative (<3.1 fmol bound estradiol/10 mg tissue) in all 8 adenomas and 4 of 5 secondary hyperplasias removed from men, and from women in 19 of 22 adenomas, 2 of 3 secondary hyperplasias, and 3 of 4 primary hyperplasias. PR were negative (<10.1 fmol bound progesterone/10 mg tissue) in 7 of 8 adenomas and all 5 secondary hyperplasias removed from men, and from women in 20 of 22 adenomas, all 3 secondary hyperplasias, and all 4 primary hyperplasias. For immunocytochemical studies, quick-frozen specimens were analyzed with a monoclonal antibody (Abbott Laboratory) directed at nuclear ER. All eight samples—five adenoma and three primary hyperplasia—were negative. We conclude that abnormal human parathyroid tissues have nondetectable levels of ER and PR. It is unlikely that estrogen and progesterone exert a direct, ER, or PR-mediated effect upon parathyroid tissue.

Key words: Estrogen receptor – Progesterone receptor – Parathyroid – Hyperparathyroidism.

Administration of estrogens and/or progestins has been reported to lower serum calcium levels in postmenopausal women with mild hyperparathyroidism [1–5]. The mechanism(s) by which these hormones alter calcium levels is not clear, as there is no agreement on whether their administration affects circulating levels of immunoreactive parathyroid hormone (PTH). Studies performed by Prince et al. [1] demonstrated an appropriate increase in PTH; those by Marcus et al. [2], Selby and Peacock [3], and Boucher et al. [5] reported no change. PTH levels were not measured in Gallagher and Nordin's study [4]. *In vitro* studies with normal bovine [6] and abnormal human parathyroid tissue [7] have suggested, however, that estrogen and progestins are both secretagogues for PTH. In an attempt to elucidate the mechanism of action in *in vivo* estrogen-induced amelioration of hypercalcemia, we examined abnormal human parathyroid tissue removed at surgery, tissue responsible for hyperparathyroidism, for the presence of estrogen and progesterone receptors. Should estrogen receptors not be present in this tissue, it would be unlikely that estrogen exerts a direct effect upon parathyroid tissue.

We employed two methods for analysis: assay of cytosolic estrogen receptors (ER) and progesterone receptors (PR) by classical hormone-receptor binding techniques, and immunocytochemistry using a monoclonal antibody-directed nuclear ER.

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Materials and Methods

Acquisition and Management of Tissue

Abnormal human parathyroid tissue removed in the course of therapeutic surgery was employed for this study. Tissue was handled in several ways: (1) Tissue termed *fresh* was placed in the operating room in RPMI-1640 culture medium (GIBCO, Columbus, IN) on ice, blotted dry, wrapped in aluminum foil and placed on dry ice for transportation to the Michigan Cancer Foundation (MCF) (approximately a 20-minute transport), where all the estrogen and progesterone assays were performed. (2) Tissue termed *frozen* was divided into pieces of approximately 1 mm^3 in size and placed into a solution consisting of 80% (v/v) RPMI-1640, 10% (v/v) autologous serum, and 10% (v/v) dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) in plastic cryopreservation vials (CryoMed, Mt. Clemens, MI). The vials, containing approximately 100 mg tissue each were cooled either in a programmable controlled-rate freezer (CryoMed) at 1°C/minute or by placement in an ethanol bath in a −70°C freezer [8]. Following freezing, tissue was stored at −135°C. Cryopreservation in either of these ways retains extracellular calcium-mediated PTH release [9]. Vials of frozen tissue to be analyzed for estrogen and progesterone receptors were removed from the −135°C freezer and transported to the MCF on dry ice. (3) Tissue termed *fresh without media* was placed directly on dry ice in the operating room and transported under those conditions. Tissue was handled in this fashion to avoid contact with serum, DMSO, or phenol red, which has weak estrogenic properties and could interfere with competitive assays for estrogen receptors [10]. (4) Tissue termed *thioglycerol* was placed in 3 mM thioglycerol in Hanks salt solution on ice in the operating room and frozen in parallel with the same patient's *frozen* tissue and stored at −135°C. Tissue was treated with thioglycerol to prevent oxidation of estrogen receptors. Thioglycerol tissue also avoided exposure to serum, DMSO or phenol red. (5) Tissue termed *quick-frozen* was placed immediately into isopentane or on dry ice for transport and stored at −135°C until preparation for immunocytochemical analysis.

Care was taken in all cases to place specimens on ice or dry ice immediately after acquisition and to freeze samples as promptly as possible in order to preserve the highly labile ERs [11].

Fresh, fresh without media, thioglycerol, and *quick-frozen* tissues consisted of a single portion of a single parathyroid gland. Vials of *frozen* tissue, on the other hand, contained randomly sorted

pieces but all from a single parathyroid gland. For some patients, two vials of cryopreserved tissue were examined. In these cases, both vials contained tissue exclusively from the same parathyroid gland but were assayed at different times.

Assignment of Clinical Diagnosis

A total of 38 patients (11 males, 27 females) were studied. Diagnoses were based on surgical findings and standard clinical criteria. Twenty-seven patients with a single large parathyroid gland in the absence of renal failure were termed *adenoma* patients, five patients with several large glands in the absence of renal failure were termed *primary hyperplasia* patients, and six with several large glands associated with profound renal failure were termed *secondary hyperplasia* patients.

Table 1. Characterization of estrogen and progesterone receptors for 30 patients with abnormal human parathyroid tissue

Variable	Number	Number (%) positive	
		Estrogen	Progesterone
Diagnosis			
Adenoma	22	3 (14)	2 (9)
Secondary hyperplasia	6	2 (33)	0 (0)
Primary hyperplasia	2	1 (5)	0 (0)
	30		
Sex			
Male	10	1 (10)	1 (10)
Female	20	5 (25)	2 (10)
Age ≤50	8	0 (0)	0 (0)
Age >50	12	5 (42)	2 (7)

Measurement of Steroid Receptors by Hormone-Receptor Binding

Preparation of Tissue. Parathyroid tissue and rabbit uterus (in estrous), which was used as a positive control, were homogenized by pulverization in liquid nitrogen to assure homogeneity. Pulverized tissue was kept at 0–4°C and homogenized in TED buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.5) using a Willems Polytron (Brinkman Instruments, Westbury, NY) twice for 15 seconds at a setting of 4 with an interval of 60 seconds. After centrifugation of the homogenate at 3500 × *g* for 15 minutes, the supernatant was diluted with buffer to a volume of 5 ml. The diluted supernatant was spun at 105,000 × *g* for 60 minutes in an ultracentrifuge (Beckman Instruments, model L5-50, Fullerton, CA) using an SW/50.1 rotor.

Estrogen Receptor Analysis. Estrogen receptors were analyzed as previously described [12]. A 2.5-ml portion of the supernatant was diluted to 5.5 ml with TED buffer (final concentration 18 mg tissue/ml), divided into 10 0.5-ml samples, and incubated at 4°C for 2 hours with tritiated 17-β-estradiol [(^{3}H)-E_2), 2,4,6,7,-^{3}H(N)-estradiol-17β], specific activity 115 Ci/mmol (E.I duPont de Nemours and Co, Boston MA) with and without 200-fold excess unlabeled E_2. Five (^{3}H)-E_2 concentrations were employed which ranged between 0.09 nM and 1.40 nM. After the incubation, 0.5 ml of dextran-coated charcoal (DCC, 1 part dextran to 10 parts charcoal) solution in TED buffer, pH 8.0 was added to each sample. Each tube was mixed, allowed to stand for 10 minutes, and then centrifuged at 2,000 × *g* for 10 minutes at 4°C. A 0.25-ml aliquot from each sample was counted in 4 ml ethanol plus 10 ml scintillation solution (8 g 2,5-diphenyloxazole and 0.2 g 1,4 bis (4-methyl-5-phenyloxazoly)-benzene in 2 liters toluene) with a liquid scintillation spectrometer equipped to correct quenching. The specifically bound (^{3}H)E_2 is defined as the difference in radioactivity in samples incubated with (^{3}H)E_2 plus unlabeled E_2 and that in samples with (^{3}H)E_2 alone. The binding capacity and dissociation constant (K_d) were determined by Scatchard plot; linear regression analysis was performed on the data. Cytosols which yielded a linear Scatchard plot with a calculated K_d between 5×10^{-9} and 10^{-10} mol/liter and a binding capacity ≥3.1 fmol estradiol-17β/10 mg, wet weight, of tumor tissue were considered ER-positive.

Progesterone Receptor Analysis. PRs were analyzed as previously described [12]. Cortisol was added to the remaining 2.5 ml of supernatant to a concentration of 3.3 μM. This sample was then divided into 0.2-ml samples and incubated for 2 hours with tritiated progesterone [(^{3}H)-Pg), 1,2,6,7-^{3}H-(N)-progesterone], specific activity 114 Ci/mmol (E.I duPont de Nemours, Boston, MA) with and without 200-fold excess unlabeled progesterone. The (^{3}H)Pg concentrations ranged between 0.45 and 7.2 nM. After incubation, 0.2 ml of TED glycerol buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 60% glycerol, pH 7.5) was added. Samples were vortexed and incubated for an additional hour. A 0.4-ml aliquot of DCC(1:10) in TED-30% glycerol buffer was then added. The tubes were mixed intermittently for 30 minutes followed by centrifugation at 4°C at 2000 × *g* for 10 minutes. The radioactivity in a 0.25-ml aliquot was determined as described above. Results were analyzed using Scatchard plots and subjected to linear regression analysis. Specimens with a binding capacity of 10.1 fmol or more progesterone/10 mg tissue were considered PR positive.

Patients with repeated assays of PR from the same gland were considered positive if any of the assays were positive.

Measurement by Immunocytochemistry

Specimens were removed from storage at −135°C and placed in TISSUE-TEK freezing matrix (Miles, Elkhart IN). Five-micron sections were cut and placed on microscope slides (Fisher Scientific, Pittsburgh PA) without tissue adhesive. Fixation and storage were performed as described in the Abbott ER-ICA Monoclonal (Abbott, North Chicago, IL) kit instructions. Slides were counterstained with hematoxylin and mounted in Permount (Fisher, Pittsburgh, PA). Positive controls employed were Abbott ER-ICA ER Positive Control Slides (Abbott) and, because we studied tissue slices rather than cell suspensions, sections of clinical breast cancer positive for ERs.

Results

Parathyroid tissue from patients of both sexes reflecting the common parathyroid diagnoses as examined (Table 1). Initially we examined cryopreserved ("frozen") tissue previously collected and readily available in a cryopreservation "bank." Because the majority of these specimens were negative for ER and PRs we began to collect specimens in additional ways which would minimize possible artifactual loss of these receptors. We completed this study with a group of nine consecutive patients, all with adenomas, all of whose tissue was collected in thioglycerol and avoided contact with serum, DMSO, or phenol red. Results appear in Tables 1 and 2. Thirty-six of 42 specimens were negative for ER. Six specimens (three adenomas, two secondary hyperplasias, one primary hyperplasia) were positive for ERs with values of 58.1, 11.4, 6.6, 7.4, 26.8, and 14.5 fmol/10 mg tissue, respectively. Four of the six positive samples were from patients who had two or three assays of the same gland, and in each of the four patients, all other assays of ER were negative. Two of these six positive specimens were below 10 fmol/10 mg tissue and could be considered "borderline positive." All 14 thioglycerol specimens collected from nine patients were negative for ERs.

Thirty-nine of 42 specimens were negative for progesterone receptors. One of the three positive specimens (all ade-

nomas) measured 10.4 fmol/10 mg tissue, just barely greater than negative; the others measured 23.7 and 107.1 fmol/10 mg tissue. Only one of the three progesterone-positive specimens was also positive for ERs.

We analyzed the sample masses by category of estrogen receptor positive and negative to determine whether negative results might be associated with smaller sample masses. There were no statistically significant differences in sample mass (Table 3). The correlation between sample mass and quantitative estrogen receptor was -0.23 ($P > 0.05$).

None of the samples from eight patients' tissue studied immunocytochemically were positive for ERs.

Discussion

Administration of estrogen and progesterone has been reported to reduce serum calcium in postmenopausal women with primary hyperparathyroidism [1–5]. The mechanism of action is not known. Prince et al. [1] have concluded that reductions in serum calcium can be explained by nonparathyroid factors, but Selby et al. [3] and Boucher et al. [5] have suggested that in view of a decrease in serum calcium without a concomitant increase in PTH, an effect upon parathyroid tissue (change in set point) is likely. *In vitro* studies, however, one with normal bovine tissue [6] and one with pathologic human tissue [7], demonstrated that estrogens and progesterone rapidly (within 1 hour) augmented PTH release. With abnormal human tissue, an effect was apparent with 10^{-9} M β-estradiol (increasing with concentrations up to 10^{-6} M) and with 10^{-7} M progesterone. Studies with normal bovine tissue demonstrated a secretagogue effect with 5×10^{-5} M of estradiol and 10^{-9} M progesterone. In the latter study, effects were seen as well with 10^{-6} M estrone, 10^{-6} M cortisol, but not with 10^{-7} M α estradiol, 10^{-6} M testosterone, and 10^{-7} M 20-α hydroxy progesterone. Addition of 10^{-7} M tamoxifen did not inhibit 10^{-7} M-estradiol-stimulated PTH release. These authors concluded that β-estradiol and progesterone acted in a specific fashion upon parathyroid tissue but probably not via conventional intracellular cytosolic receptors in view of the rapidity of response and failure of tamoxifen to inhibit the response.

Table 2. Measurement of estrogen and progesterone receptors in 42 specimens of abnormal human parathyroid tissue from 30 patients

Variable	Number	Number (%) positive	
		Estrogen	Progesterone
Diagnosis			
Adenoma	30	3 (10)	3 (10)
Secondary hyperplasia	8	2 (25)	0 (0)
Primary hyperplasia	4	1 (25)	0 (0)
	42		
Sex			
Male	13	1 (8)	1 (8)
Female	29	5 (17)	2 (7)
Age ≤50	9	0 (0)	0 (0)
Age >50	20	5 (25)	2 (10)
Sample management[a]			
Fresh	6	2 (33)	2 (33)
Fresh w/o media	5	2 (40)	0 (0)
Frozen	17	2 (12)	0 (0)
Thioglycerol	14	0 (0)	1 (7)
	42		

[a] Sample management: fresh = in media, not cryopreserved; fresh w/o media = immediately on dry ice; frozen = cryopreserved in media/DMSO/serum, kept at −135°C; thioglycerol = in 3 mM thioglycerol/Hanks salts, kept at −135°C

Table 3. Descriptive statistics for sample mass by type of receptor

Condition	N	Mass of samples		
		Mean	SD	Range
ER positive	6	292	323	60–901
ER negative	36	687	565	41–2876
PR positive	3	251	232	95–518
PR negative	39	660	561	41–2876

We have directly measured ER and PRs in tissue responsible for hyperparathyroidism. Specimens were obtained from both men and women and from patients with adenoma, secondary hyperplasia, and primary hyperplasia. We initially used tissue frozen in a manner known to preserve calcium-mediated PTH release but when so few specimens were positive, we examined specimens collected in a variety of ways. Because of concern that phenol red could act as an estrogen [10] and mask ERs, we collected some specimens in the absence of media. Because of concern that estrogen receptors could be slowly oxidized during long-term storage, we collected some specimens in 3 mM thioglycerol to inhibit oxidation. Overall, only 6 and 3 of 42 specimens were positive for ER and PRs, respectively. Results varied on occasion among specimens from the same patient but there appeared to be no consistent effect of tissue handling upon results. It may be that estrogen and progesterone receptors, if present in human parathyroid tissue, are few in number, unevenly distributed, or both. Additional studies employing immunocytochemical analysis failed to demonstrate the presence of estrogen receptor-positive cells in specimens from eight additional patients.

In vivo studies of exogenous estrogen [4, 13–16] in postmenopausal women and of the normal fluctuations of estrogen and progesterone in menstruating women [17, 18] have failed, with one exception [1], to demonstrate a correlation with peripheral blood PTH concentrations.

Our observations taken with the *in vivo* and *in vitro* studies of other investigators suggest that estrogen and progesterone probably do not act directly upon human parathyroid tissue, at least not via conventional ER and PR. Under *in vitro* conditions and at supra-physiologic concentrations, these hormones augment PTH release but by undefined mechanisms.

It is probable that estrogen-induced changes in serum calcium are mediated by changes in bone physiology. These changes may be a consequence of direct effects of estrogen upon bone. ERs have been identified on human bone cells although at low concentration [19]. Alternatively, (additionally?) estrogen may exert indirect effects upon bone via calciotropic hormones other than PTH. Stevenson et al. [20] has demonstrated an increase in calcitonin as a consequence of estrogen administration.

The observation that serum calcium falls in postmenopausal women with hyperparathyroidism taking estrogen is unlikely to reflect a direct effect of estrogen upon the intrinsic calcium regulatory defect characteristic of hyperparathyroidism.

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