Effects of Estradiol on Estrogen Receptor, Progesterone Receptor, and Tyrosinase in Hamster Melanoma Transplanted into Athymic Mice

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ABSTRACT

Nuclear estrogen binding was characterized in HM-1, a malignant hamster melanoma cell line transplanted into male and female athymic mice following acute, subchronic, and chronic exposure of estradiol. Nuclear binding was saturable, of high affinity (10^8 M^-1) and readily soluble in low salt buffer. Saturation analysis revealed that [3H]estradiol in excess of 5.0 nM is apparently bound to a second class of lower affinity (10^4 M^-1), with a specific monoclonal antibody (H222 Spy) directed against the human estrogen receptor protein. In contrast, estrogen receptor protein was in excellent agreement (r = 0.93) with values obtained using hydroxypaptate to separate bound from free ligand.

Nuclear estrogen receptor content in HM-1 cells was increased maximally 1 h after acute s.c. injection of a low dose (0.1 µg) of estradiol. The increase in nuclear receptor content was accompanied by an apparent rapid reduction in cytosol binding. Subchronic (3 days) and chronic exposure (35 days) to estradiol also produced a significant, dose-related increase in tumor nuclear estrogen receptor content.

Cytosol binding for progesterin was low (<2 fmol) absent in HM-1 xenografts not exposed to estradiol. Subchronic and chronic exposure to estradiol induced a dose-related, specific, high affinity (10^8 M^-1) cytosol binding protein for progesterin. In HM-1 xenografts carried in male and female athymic mice, progesterone binding to nuclear receptor was not increased in estrogen-primed animals, nor did acute injection of progesterone (100 µg s.c.) increase the amount of saturable, high affinity (10^8 M^-1) nuclear progesterone receptor in control or estradiol-primed athymic mice.

In contrast to the induction of progesterin binding, tyrosinase activity was not altered by a similar exposure to estradiol when assayed at a saturating concentration of tyrosine. These observations suggest that the estrogen receptor in HM-1 cells may be functional but that pigmentary changes observed in mammals following chronic exposure to estradiol may not be mediated by a direct effect on the rate limiting enzyme of melanin synthesis.

INTRODUCTION

We have recently suggested that the transplantable, malignant hamster melanoma cell line HM-1 contains a specific, high affinity cytosol receptor for estrogen (1) and may provide a useful model for investigating the putative relationship between melanocytes and gonadal steroids (2–9). In addition to a uniform sex-dependent response in growth to castration and estrogen replacement in syngeneic hosts (10, 11) and in vitro (1, 12), subclones of the original heterologous, ER^- hamster melanoma cell line MM1 are also ER^+ and exhibit rapid sex-dependent growth when transplanted into athymic mice (1, 13, 14).

The mechanism(s) underlying estrogen induced alterations in melanoma growth and pigmentation remain controversial. The binding protein exhibits several characteristics of ER in other estrogen responsive tissues (1) but has not as yet been shown to be functional in either melanocytes or melanoma from any species. Although pharmacological concentrations of estradiol inhibit HM-1 growth in vitro (1, 12) suggesting a direct, possibly receptor-mediated effect, at least one report (15) suggests that estimates of ER in human melanoma using a dextran-coated charcoal assay are erroneous and result from the generation of tritiated water from the [3H]estradiol substrate by tyrosinase, the rate limiting enzyme in melanin synthesis (16). Additional evidence suggests that ER in melanoma can be reliably estimated if sulfhydryl reducing agents are incorporated into the assay buffer and hydroxypaptate, rather than dextran-coated charcoal, is used to separate bound from free ligand (1, 17). Recent evidence also suggests that ER, as identified by enzyme-linked immunoassay and isoelectric focusing is present in a significant number of primary and metastatic melanomas in humans (18).

Progesterone has been reported to both stimulate melanogenesis at the expense of human melanocyte growth in vitro (19) and enhance murine melanoma growth in serum-free media (20, 21). Estrogen is known to specifically induce receptor for progesterone in responsive cells (reviewed in Ref. 22). It remains, therefore, to demonstrate that estrogens induce the synthesis of specific proteins in pigmented cells, and whether estrogens alter pigmentogenesis and cell proliferation directly or indirectly.

The use of xenografts in athymic mice to investigate hormonal effects on melanoma biology provides a model with reduced levels of circulating estrogen(s) (23) and avoids species differences in hormonal or immune environment, particularly steroidal effects on thymic lymphocytes (24) which may partially mask any estrogenic effects on melanoma behavior in vivo. The present study describes the binding of estradiol to nuclei of HM-1 melanoma xenografts following exposure of male and female athymic mice to estradiol and associated changes in cytosol and nuclear progesterone receptor content and tyrosinase activity.

MATERIALS AND METHODS

Reagents. [2,4,6,7^-3H]Estradiol-17β (specific activity, 90–115 Ci/mmole) 17-α-methyl-^-3H-labeled R5020 (specific activity, 70–87 Ci/mmole). The abbreviations used are: ER, cytosol estrogen receptor; ER, nuclear estrogen receptor; PgR, progesterone receptor; PgR, nuclear progesterone receptor; MEM-H, minimum essential medium with Hanks’ salts; TES, N-tris(hydroxymethyl)methyl-1-aminoethane sulfonic acid; EIA, enzyme immunoassay; HAP, hydroxypaptate; R5020, promegestone, 17α methyl R5020.
mmol), radioiinert R520, and t-[3,5-3H]tyrosine (specific activity, 40–60 Ci/mmol) were purchased from New England Nuclear Co. (Cambridge, MA). Additional radioiinert steroids were purchased from Sigma Chemical Co. (St. Louis, MO) as were t-tyrosine, t-dopa, calf thymus DNA, hydroxyapatite, Tris HCl, TES, phenylmethylsulfonylfluoride, N-ethylmaleimide, EDTA, Triton X-100, sodium azide, sodium molybdate, and benzamidine. Crystalline bovine serum albumin was obtained from Armour and Co. (Chicago, IL). Activated charcoal was obtained from J. T. Baker Co. (Phillipsburg, NJ) and Dextran T-70 from Pharmacia (Piscataway, NJ). Estradiol pellets (0.1, 0.5 mg) in a timed release matrix of cholesterol: methyl cellulose: lactose (vehicle control) were purchased from Innovative Research of America (Rockville, MD). Protein reagents were obtained from Bio-Rad Laboratories (Richmond, CA) as was the Dowex cation exchange resin (50W x 12, 200–400 mesh). Media, antibiotics, fetal bovine serum, and trypsin-EDTA solution were purchased from GIBCO Laboratories (Grand Island, NY). The ER-EIA kit containing a specific monoclonal antibody to human estrogen receptor (H222 Spy) was a gift from Abbott Laboratories (North Chicago, IL).

Cell Line. HM-1 was subcloned in our laboratory (1) from a spontaneous hamster melanoma (MM1) (25) provided by Dr. J. Fortner (Memorial Sloan-Kettering Institute) and maintained in minimum essential medium with Hanks' salts supplemented with 16% fetal bovine serum and antibiotics. HM-1 cells (5 x 10^6) in 0.1 ml minimum essential medium with Hanks' salts were inoculated s.c. in the right and left flanks of 4–to 5-week-old male or female athymic mice as previously described (1).

Athymic Mice. BALB/c derived athymic (nu/nu) mice (National Cancer Institute, Frederick, MD, courtesy of Dr. C. Reeder) 4–5 weeks of age were used. Mice were maintained as previously described (1, 6). Acutely treated mice were given injections s.c. of estradiol in 6% ethanol/0.9% saline solution (0.1, 2.5, or 10 mg/0.1 ml), progesterone in 20% ethanol/0.9% saline solution (100 mg/0.1 ml), or vehicle alone during log phase growth and killed 1, 2, 6, or 24 h later. Subchronically treated mice received injections (10 mg/kg) twice or three times 3 days with the final injection 12 h prior to sacrifice. Mice were either sham gonadectomized or bilaterally gonadectomized under the same conditions 1 week prior to s.c. implantation of estradiol or vehicle pellets. The pellets were implanted 1 day prior to s.c. inoculation of HM-1 cells. Additional pellets were reimplanted 21 days later to maintain elevated plasma levels of estradiol. At necropsy, blood was collected by cardiac puncture and plasma was separated and stored at −20°C. All flanks tumors were carefully excised, trimmed of any necrosis, rinsed in phosphate-buffered saline, blotted dry, and immediately frozen prior to receptor assay. All statistical procedures were carried out using the BMDP series of programs (BMDP Statistical Software, Los Angeles, CA) on a PDP-11 computer.

Estradiol Receptor Assay. Tumor cytosols were prepared in 10 mM Tris-HCl-1.5 mM EDTA buffer under the same conditions. The remainder of the assay was carried out as previously described (33) over a concentration range of 0.2–3.0 nM [H]-labeled R520. A single saturating concentration of 3.0 nM [H]-labeled R520 was used to routinely assay PgRc content.

Ligand Specificity. Cytosol aliquots of HM-1 (105,000 x g supernatant, 200 µl) were incubated at 0°C for 2 h with 5.0 nm [H]-labeled R520 in the presence of increasing concentrations of radioiinert steroids. Bound and free ligands were separated with dextran-coated charcoal (0.5% charcoal-0.05% dextran T-70 in 10 mM Tris-HCl-1.5 mM EDTA, pH 7.4). One hundred % specifically bound was calculated by subtracting the difference in total [H]-labeled R520 bound from that bound in the presence of 5 µM radioiinert R520.

Tyrosinase Assay. The tyrosinase assay was modified slightly from the method of Townsend et al. (34). Melanoma specimens identical to those used in the ER-EIA were homogenized in three volumes of tyrosinase buffer (236 mM sucrose, 10 mM KCl, 6.67 TES, 3.32 mM Tris-base, 2.0 mM phenylmethylsulfonylfluoride, 1.0 mM EDTA, 1.0 mM N-ethylmaleimide, 1.0 mM benzamidine, and 0.2% Triton X-100, pH 7.4) (34) at 0–4°C in a glass homogenizer with 20 strokes of a Teflon pestle and fractionated according to the method of Seiji et al. (35). HM-1 cells (200-µl aliquots, 400 x g supernatant) were incubated at 37°C with 10 µl air-dried aliquots of purified [H]tyrosine, equivalent to 5000 dpm, 50 µl of stock L-tyrosine (6 mM L-tyrosine-1,7.6 mM TES, pH 6.5; 20 µl 6.96 mM L-dopa-33.4 mM TES-0.97 mM Tris, pH 6.0) to yield final concentrations of 1.1 and 0.52 mM, respectively, which maximized tyrosinase activity (34, 36). The remainder of the assay was carried out essentially according to the procedure of Townsend et al. (34).

Rate constants were obtained by varying the amount of radioiinert L-tyrosine from 25 nmol/270 µl (0.092 HIM) to 300 nmol/270 µl (1.1 HIM). Higher concentrations were not soluble. Since tyrosinase is inhibited if the concentration of L-dopa is high relative to L-tyrosine, the amount of L-dopa was varied to maintain a constant percentage, 46.4%, of that of Boomsma et al. (33). Frozen pulverized melanoma specimens were suspended in 5 volumes of PgR buffer (0.5 mM sucrose-2 mM MgCl2-10 mM Tris-HCl-0.02% NaN3, pH 7.4) and homogenized at 0–4°C (33). All subsequent procedures were carried out at the same temperature. After centrifugation at 1,300 x g for 10 min to remove the nuclear pellet, the supernatant sol was recentrifuged at 105,000 x g for 1 h and cytosol aliquots (200 µl) were incubated for 2 h with [H]-labeled R520 (0.2–5.0 nM) and a 250-fold molar excess of unlabeled cortisol with or without a 250-fold molar excess of unlabeled R520. Bound and free R520 were separated by the addition of 300-µl aliquots of dextran-coated charcoal (0.5% charcoal-0.05% dextran T-70 in 10 mM Tris-HCl-1.5 mM EDTA, pH 7.4), incubated for 5 min, centrifuged at 1,800 x g for 10 min, and the supernatant sol counted in 8.0 ml ACS scintillation cocktail (Beckman Instruments, Palo Alto, CA). Single-point binding assays were performed under the same conditions using a saturating (3.0 nM) concentration of [H]-labeled R-520. For assay of PgRc, the pellet was resuspended in assay buffer (same as for PgR) with 30% glycerol, pH 7.4, at 0–4°C, rehomogenized, recentrifuged at 1,300 x g for an additional 10 min, and washed again in assay buffer under the same conditions. The remainder of the assay was carried out as previously described (33) over a concentration range of 0.2–3.0 nM [H]-labeled R520. A single saturating concentration of 3.0 nM [H]-labeled R520 was used to routinely assay PgRc content.

Radioimmunoassay. Estradiol was quantitated with an antibody generated against estradiol-6-carboxymethyloxyime bovine thyroglobulin (courtesy of Drs. K. Wright and D. C. Collins, Emory University) at a final antibody dilution of 1:30,000 at which there is negligible (<1%) cross-reactivity to estrone or estriol. Coefficients for inter- and intrassay variations for eight paired replicates of a standard solution of estradiol (20 pg/ml) were 7.2 and 8%, respectively.

Progesterone Radioimmunoassay. Progesterone was quantitated using an antibody generated against 17-hydroxyprogesterone 3-oxime (courtesy of Dr. Max Amoss, Texas A & M University) at a final antibody dilution of 1:15,000. Appropriate cross-reactivity with 11α-hydroxyprogesterone and 17α-progesterone. The coefficients for inter- and intrassay variations for eight paired replicates of a pooled-
RESULTS

Plasma Estradiol and Progesterone Levels. One h after male and female athymic mice received an acute injection of 2.5 μg estradiol, plasma estradiol levels were 33- and 16-fold higher than control, respectively, and remained 10- to 14-fold higher than controls 24 h postinjection. The increases were dose related (Table 1). Plasma estradiol levels in male mice that received subchronic injections of 10.0 μg estradiol increased 5-fold over vehicle injected controls (16.8 ± 4.5 (SE) pg/ml, N = 20; 84.5 ± 6.9 pg/ml, N = 21; P < 0.01). Plasma estradiol levels in mice given implants of 0.1 and 0.5 mg estradiol pellets were significantly increased over vehicle controls in intact male (16.8 ± 4.5, N = 20; 26.7 ± 4.8, 0.1 mg, N = 5; 92.7 ± 8.8, 0.5 mg, N = 5), intact female (17.8 ± 3.1, N = 7; 104.6 ± 28.9, 0.5 mg, N = 6), and Ovax female (12.9 ± 2.6, N = 8; 90.4 ± 12.6, 0.5 mg, N = 7; 192.2 ± 47.4, 0.5 mg, N = 5) mice. The 0.5-mg pellet increased plasma estradiol significantly in all cases and represents the upper limit of normal for the preovulatory increase in estradiol in heterozygous littermates (23). Plasma progesterone levels (ng/ml) were increased (P < 0.001) 1 h after s.c. injection of 100 μg progesterone (males, 2.1 ± 0.2, N = 42; 37.3 ± 3.7, N = 9; females, 1.9 ± 0.3, N = 18; 36.9 ± 5.8, N = 22).

Estrogen Receptor. Saturation analyses of HM-1 cytosols indicated that a [3H]estradiol concentration of ~2.0 nM saturated specific (1), high affinity, low capacity sites in tumors maintained in mice of either sex (Fig. 1; Table 2). Concentrations of estradiol in excess of 5.0 nM appeared to bind to a second class of lower affinity higher capacity (>10 fmol/mg protein) sites (Fig. 1). There was no sex-related difference in ER, affinity or capacity (Table 2).

Specific binding of [3H]estradiol to an HM-1 nuclear fraction was maximal at 30°C for 35 min; ERc levels rapidly decreased after 15 min at 37°C (data not shown). High affinity nuclear sites were saturated at 3.0 nM estradiol with no sex-related difference in affinity (Kd) or content (Table 2). Binding of ligand was apparently specific for estrogen(s) as the regression coefficient for ER-EIA values and those obtained for ER, using HAP to separate bound from free ligand was >0.90 (Fig. 2). The relatively low capacity of receptor for estradiol did not appear to significantly influence either the accuracy or precision of the ER-EIA. Since the ER-EIA buffer contains molybdate ion, significant correlation with the HAP assay also suggests minimal degradation of receptor in HM-1.

ER, in HM-1 carried in male mice was increased relative to controls in response to the elevated blood levels of estradiol 1 h postinjection of 0.1, 2.5, or 10 μg estradiol. The increase was maximal at 0.1 μg estradiol (Fig. 3). ER, and ERc content in HM-1 in mice given injections of 2.5 μg estradiol or vehicle

![Table 1 Plasma estradiol concentration of male and female athymic mice with time after acute injection of estradiol](image)

![Fig. 1. Scatchard analysis of specific ER binding in HM-1 cytosol.](image)

![Fig. 2. Correlation of ER-EIA values with results obtained by HAP assay and Scatchard analysis on identical tumors.](image)
ER IN HAMSTER MELANOMA

Fig. 3. ER content of HM-1 from male athymic mice 1 h after s.c. injection of estradiol. ERc and ERn values are different (P < 0.01; ANOVA-Student-Newman-Keuls multiple comparison test) from pretreatment levels. Single point exchange assays were done at 30°C with 3.0 nM [3H]estradiol. Points, three individual tumors; bars, SE; fmol.

Table 3 Estrogen receptor content of HM-1 xenografts with time after acute s.c. injection of estradiol

<table>
<thead>
<tr>
<th>Sex</th>
<th>Estradiol (µg)</th>
<th>h after estradiol</th>
<th>ERc</th>
<th>ERn</th>
<th>Total (ERc + ERn) (mean)</th>
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</thead>
<tbody>
<tr>
<td>M</td>
<td>0.0</td>
<td>0</td>
<td>18.6 ± 2.5*</td>
<td>2.3 ± 0.9*</td>
<td>20.9 ± 3.4</td>
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<td></td>
<td>2.5</td>
<td>1</td>
<td>7.3 ± 0.6*</td>
<td>28.7 ± 8.3*</td>
<td>35.0 ± 9.9</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>12.5 ± 2.9</td>
<td>14.6 ± 1.5</td>
<td>27.1 ± 4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>17.5 ± 3.3</td>
<td>11.2 ± 1.6</td>
<td>28.7 ± 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>18.2 ± 5.3</td>
<td>13.7 ± 2.4</td>
<td>31.9 ± 7.7</td>
</tr>
<tr>
<td>F</td>
<td>0.0</td>
<td>1</td>
<td>19.4 ± 3.8</td>
<td>10.1 ± 1.0</td>
<td>29.5 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1</td>
<td>4.8 ± 1.5*</td>
<td>25.7 ± 4.2*</td>
<td>30.5 ± 5.7</td>
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<td>9.3 ± 2.6</td>
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<td>18.8 ± 6.5</td>
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<td></td>
<td></td>
<td>24</td>
<td>18.1 ± 6.4</td>
<td>12.5 ± 5.4</td>
<td>30.6 ± 8.9</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Table 4 Estrogen receptor content of HM-1 xenografts from intact female and ovariectomized mice after subchronic (10 µg/3 days) and chronic exposure to estradiol

<table>
<thead>
<tr>
<th>Surgical treatment</th>
<th>Exposure</th>
<th>Estradiol dose (µg)</th>
<th>ERc 4°C</th>
<th>ERc 30°C</th>
<th>ERn 30°C</th>
<th>Total (ERc + ERn) (mean at 30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F Intact</td>
<td>Subchronic</td>
<td>0.0</td>
<td>23.1 ± 5.3* (5)</td>
<td>12.0 ± 5.3 (1)</td>
<td>10.9 ± 1.6 (3)</td>
<td>27.4</td>
</tr>
<tr>
<td>F Intact</td>
<td>Chronic</td>
<td>0.0</td>
<td>8.0 ± 2.0* (5)</td>
<td>15.7 ± 3.8 (3)</td>
<td>23.6 ± 4.1* (3)</td>
<td>39.3</td>
</tr>
<tr>
<td>F Ovariectomized</td>
<td>Subchronic</td>
<td>0.0</td>
<td>25.2 ± 5.9 (3)</td>
<td>29.5 ± 4.9 (3)</td>
<td>10.2 ± 1.0 (3)</td>
<td>39.7</td>
</tr>
<tr>
<td>F Ovariectomized</td>
<td>Chronic</td>
<td>0.1</td>
<td>12.2 ± 3.0* (3)</td>
<td>12.3 ± 0.2* (3)</td>
<td>15.8 ± 3.4* (3)</td>
<td>28.1</td>
</tr>
<tr>
<td>F Ovariectomized</td>
<td>Chronic</td>
<td>0.5</td>
<td>11.0 ± 2.1* (3)</td>
<td>11.5 ± 3.1* (3)</td>
<td>18.9 ± 1.8* (3)</td>
<td>30.4</td>
</tr>
</tbody>
</table>

* Mean ± SE.

DISCUSSION

The present study supports initial observations of a specific ER in HM-1 cytosols (1) and further suggests that HM-1 exposure to estradiol decreased ER content (P < 0.05) and increased ERn (P < 0.05) in HM-1 similarly to that observed in HM-1 from mice treated subchronically with estradiol (Table 4). The total amount of estrogen receptor (assayed at 30°C) did not increase following subchronic or chronic exposure to estradiol.

Progesterone Receptor. PgRn content was barely detectable (<2 fmol/mg protein by single point assay) in HM-1 cytosol from untreated mice. Acute treatment with 2.5 µg estradiol did not increase PgRn content (fmol/mg protein) 24 h postinjection s.c. (males, 1.0 ± 0.1; N = 3; 1.5 ± 0.6; N = 3; females, 1.0 ± 0.3; N = 3; 1.5 ± 0.4; N = 3). However, subchronic exposure to estradiol increased HM-1 PgRn content 9- to 21-fold relative to respective controls without significantly altering PgRn (Table 5). Chronic treatment of male mice with estradiol produced a dose-related increase in PgRn as measured by single point assay with no significant alteration in PgRn (Table 5). Removal of the ovaries had no effect on the ability of estradiol to induce PgRn (data not shown). Cytosol and nuclear binding for progesterone was not dependent on sex, specific (Fig. 4), saturable (~2-3 nM), and of high affinity (Figs. 5 and 6; Table 6). There was no evidence of lower affinity binding in either cytosol or nuclear fractions. Since chronic exposure to estrogen had no apparent affect on PgRn, we investigated whether progesterone could induce its own receptor in HM-1 in the presence and absence of estrogen priming. Acute injection of progesterone (100 µg s.c.) had no effect on PgRn or PgRn levels 1 h posttreatment even in estradiol-primed mice. Additional determinations of PgRn at 0.5 and 2 h postinjection showed no change in binding (data not shown).

Tyrosinase. Michaelis-Menten analysis of tyrosinase activity in subfractions of HM-1 tumors identical to those on which receptor assays were performed showed that maximal enzyme activity was obtained at 1.1 mM tyrosine at 37°C. At this substrate concentration and temperature tyrosinase activity was linear over a broad range of protein concentrations (1–15 mg/ml) up to 4 h. Lineweaver-Burke analysis of tyrosinase activity suggested that the K(M) (males, 576.7 ± 86.7; females, 559.5 ± 43.8 µM), V(max) (males, 3.6 ± 0.5; females, 3.3 ± 0.2 nmol/h/mg protein), and V(300) (males, 2.9 ± 0.2; females, 2.5 ± 0.2 nmol/h/mg protein) values were similar in HM-1 tumors from untreated mice of both sexes. When the tyrosine concentration was not rate limiting and the molar ratio of tyrosine to L-dopa kept constant (see "Materials and Methods"), there was no change in V(300) after acute, subchronic, or chronic exposure to estradiol (Table 7).

DISCUSSION

The present study supports initial observations of a specific ER in HM-1 cytosols (1) and further suggests that HM-1...
nuclei express a saturable, high affinity binding component for estradiol. Lower affinity, higher capacity binding sites also appear to be present in the cytosol fraction of HM-1 cells. This may represent the type II binding sites previously reported for other estrogen responsive tissues and may provide a means for maintaining tumor responsiveness after subchronic or chronic exposure to estradiol (38).

Estrogen receptor in HM-1 cells is also bound by a specific monoclonal antibody which recognizes the binding protein in a number of species (39). The excellent correlation with values obtained using HAP and minimal background level of binding (≤1 fmol/mg cytosol protein) with the monoclonal antibody to ER provides further suggestive evidence that ER in this tumor is similar to that of other estrogen responsive tissues. Previous unsuccessful attempts to identify receptor for estrogen in frozen sections of human melanoma by immunocytochemical means (40, 41) might have resulted from the relatively low levels of receptor in unstained tissue and the relative insensitivity of the ER-ICA at low levels of receptor (42). Although castration of either sex did not significantly alter HM-1 ERc and ERn contents from control, the rapid changes in high affinity ERc and ERn contents in HM-1 cells after acute injection of estradiol appear to be similar to those observed in other estrogen responsive tissues (43). The lack of effect of castration on ER content may in part be due to the abnormally low levels of estradiol in these homozygous mice which, unlike their heterozygous littermates, do not exhibit an estrous cycle and are not fecund (23). Interestingly, while acute, subchronic, and chronic presentation of estradiol apparently increased tumor responsiveness, the final increase in ERc may in part be due to either a decrease in the amount of occupied ERc. Unlike other responsive tissues (43, 44) the rapid increase in ERc following a maximal decrease at 1 h suggests that the increase in ERc may in part be due to changes in the amount of free ER. The lack of effect of castration on ERc content without altering affinity there was no corresponding increase in ERn. Total receptor content remained similar in all treatment groups. This finding is in sharp contrast to previous observations of a significant increase in protein synthesis and ER, in rat uterus after an acute injection of estradiol (44). Katzenellenbogen (45) has suggested that the increase in estrogen receptor population may be part of the molecular mechanism by which responsive tissues become increasingly more sensitive to estrogen. Although ERc was not elevated above control at 24 h the steady increase in ERc content to pretreatment levels following a maximal decrease at 1 h suggests that the rise in ERc may in part be due to either a decrease in the stability of ERc with time or reprocessing of the receptor. The short half-life and ready solubility of ERc in low salt buffer supports other possibility (46). The lack of difference in ERc levels at 4 and 30°C suggests that the return of ERc to preinjection levels is not due to a change in the amount of occupied ERc. Unlike other responsive tissues (43, 44) the rapid increase in ERc was not sustained beyond 1 h postinjection. The rapid decline in ERc, as measured by exchange, resembled that ob-

### Table 5 Progesterone receptor content of HM-1 from male and female athymic mice after subchronic and chronic exposure to estradiol

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Dose (mg)</th>
<th>PGRe</th>
<th>PGRe/</th>
<th>Total (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Subchronic</td>
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<td>4.8 ± 0.5</td>
<td>19.3 ± 3.9</td>
<td>24.1</td>
</tr>
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<td>M</td>
<td>Estriadiol</td>
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<td>42.3 ± 6.1</td>
<td>20.0 ± 2.4</td>
<td>62.3</td>
</tr>
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<td>F</td>
<td>Subchronic</td>
<td>0.0</td>
<td>3.1 ± 0.4</td>
<td>24.2 ± 2.6</td>
<td>27.4</td>
</tr>
<tr>
<td>F</td>
<td>Estriadiol</td>
<td>0.01</td>
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<td>23.2 ± 1.7</td>
<td>89.7</td>
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<td>Chronic</td>
<td>0.0</td>
<td>4.2 ± 0.4</td>
<td>24.9 ± 2.5</td>
<td>29.1</td>
</tr>
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<td>M</td>
<td>Estriadiol</td>
<td>0.1</td>
<td>31.2 ± 8.7</td>
<td>24.8 ± 3.6</td>
<td>56.0</td>
</tr>
</tbody>
</table>

*HM-1 cytosol aliquots (105,000 × g supernatant, 200 µl) were incubated for 2 h at 0°C with 3.0 nM 3H-labeled R5020 and a 250-fold molar excess of unlabeled cortisol with or without a 250-fold molar excess of unlabeled R5020. Dextran-coated charcoal (0.5% charcoal-0.05% dextran T-70 in 10 mM Tris-HCl-1.5 mM EDTA, pH 7.4) was used to separate bound from free ligand.

Fig. 4. Ligand specificity of PgRc binding in HM-1 cytosol. 3H-labeled R5020 (5 nM) and competing ligands were incubated for 2 h at 0°C. One hundred % specific binding was calculated by subtracting the difference in total 3H-labeled R5020 bound from that bound in the presence of 5 nM radioinert R5020. Fifty % inhibitor concentration values (nM) were R5020, 1.5; progesterone, 30; α-methyltestosterone, 80; dihydrotestosterone, 200.

Fig. 5. Scatchard analysis of 3H-labeled R5020 binding in HM-1 cytosols. 3H-labeled R5020 (0.2–5.0 nM) was incubated at 0°C for 2 h in the presence and absence of a 250-fold molar excess of radioinert R5020 and cortisol. Ka, 0.48 nM; B:max, 13.8 fmol (fm)/mg protein, r = 0.98. Inset, saturation analysis of 3H-labeled R5020 binding in HM-1 cytosol. Available protein sites saturated at 2.0 nM 3H-labeled R5020. B/F, bound/free.
NaOH hydrolysis was used to solubilize the nuclear receptor may also be occurring. The ERc in HM-1 cells appears as a larger species on sucrose density gradients in the presence of molybdate, but ERr content does not increase significantly (1). The synthesis of progesterone receptor is specifically regulated by estrogen (22). Induction of PgRc is a marker for estrogen receptor functionality in a number of normal (48, 49) and malignant (50, 51) target tissues. The apparent dose-related increase in PgRc after subchronic or chronic exposure to estradiol would support the concept of a functional estrogen receptor in HM-1 cells. However, in responsive tissues, estrogen priming increases PgRc as well as PgRr content (52, 53). Although PgRc content increased significantly following subchronic or chronic exposure to HM-1 cells to estradiol, there was no change in PgRr content. While estradiol is believed to induce and maintain high levels of PgRc in reproductive tract cells, an injection of progesterone also appears to translocate the receptor into the nucleus (49). If progesterone is administered following an estrogen priming, the level of PgRc in responsive cells rapidly decreases (48), with a rapid accumulation of PgRr in the nucleus (54, 55). Based on these observations, an acute dose of progesterone should also increase PgRc (and decrease PgRr content) in HM-1 cells after sufficient priming with estradiol. Interestingly, PgRc content did not change 1 h after tumor-bearing male or female mice, primed subchronically or chronically with estradiol, were given injections s.c. of 100 μg of progesterone, although plasma progesterone levels increased significantly. The progesterone receptor, apparently induced after subchronic or chronic exposure to estradiol may be “defective” in that it could possess some altered property which prevents translocation or association with acceptor sites in the nucleus (56, 57). Although thenature of the “altered property” is presently unknown, the synthesis of progesterone receptor is specifically regulated by estrogen (22). Induction of PgRc is a marker for estrogen receptor functionality in a number of normal (48, 49) and malignant (50, 51) target tissues. The apparent dose-related increase in PgRc after subchronic or chronic exposure to estradiol would support the concept of a functional estrogen receptor in HM-1 cells. However, in responsive tissues, estrogen priming increases PgRc as well as PgRr content (52, 53). Although PgRc content increased significantly following subchronic or chronic exposure to HM-1 cells to estradiol, there was no change in PgRr content. 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The progesterone receptor, apparently induced after subchronic or chronic exposure to estradiol may be “defective” in that it could possess some altered property which prevents translocation or association with acceptor sites in the nucleus (56, 57). Although the nature of the “altered property” is presently unknown, the difference is probably not due to changes in receptor affinity or specificity since binding constants agree with those reported for other tissues (58, 59) and binding was specific for progesterins. Activation of tyrosinase by estradiol might be an alternative to a direct, receptor-mediated mechanism for the growth inhibitory effect observed in vivo and in vitro (1–4). Melanoma growth reportedly is inversely related to tyrosinase activity (60), which might result from highly unstable free radical precursors to melanin generated by quinone intermediates that are cytotoxic to melanoma cells (61–63). Collectively, present results indicate that acute, subchronic, or chronic exposure to estradiol in vivo does not alter enzyme activity in HM-1 cells. It would appear that either estrogen(s) does not alter tyrosinase activity directly or its effect on pigmentation may be more complex than originally conceived. Stimulation of melanin synthesis may occurred in rat uterus following an acute injection of estradiol, where ERr was within control values by 6 h postinjection (43). The shorter residency time in HM-1 nuclei apparently does not alter the inhibitory effects of chronic exposure of estradiol on HM-1 growth (1). Long-term exposure to estrogens can produce a prolonged elevation in ERr in estrogen sensitive tissues (47). Collectively, our data suggest that after subchronic or chronic exposure to estradiol there is a sustained increase in ERr in HM-1 cells, but total receptor content does not increase. Although the sustained increase in ERr content suggests that stabilization of the estrogen receptor may have occurred in the presence of elevated levels of estradiol, the significant decrease in ERr content during chronic exposure to estradiol also suggests that some degradation of receptor may also be occurring. The ERr in HM-1 cells appears as a larger species on sucrose density gradients in the
depend on prior exposure to estrogen followed by progesterone treatment. Estrogen priming has been reported to potentiate the effect of progesterone on melanocytes (64, 65). Carruthers observed that in men progesterone causes melasma, hyperpigmentation of facial skin regions, but only if estrogen was administered simultaneously, and he postulated that progesterone acts on estrogen-primed melanocytes. After estrogen priming has induced the synthesis of progesterone receptor in the melanocyte, progesterone could act synergistically with estrogen to stimulate tyrosinase activity thereby enhancing skin pigmentation.

While the actual mechanism(s) of the inhibitory effect of estradiol on HM-1 growth (1) remains unknown, present results suggest that protein induction or repression in HM-1 cells would be a likely avenue for investigation.

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24. Dutton, D. L., Doull, K., and Beattie, C. W. Estradiol on HM-1 growth (1) remains unknown, present results suggest that protein induction or repression in HM-1 cells would be a likely avenue for investigation.

ER IN HAMSTER MELANOMA


Effects of Estradiol on Estrogen Receptor, Progesterone Receptor, and Tyrosinase in Hamster Melanoma Transplanted into Athymic Mice

M. Helen Hitselberger, Rosemary L. Schleicher and Craig W. Beattie