Inhibition of Hamster Melanoma Growth by Estrogen

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ABSTRACT

A malignant hamster melanoma cell line HM-1 derived from the heterogenous malignant hamster melanoma MM1 contains a specific, high affinity binding protein for estrogens. Partial purification of the binding protein with ammonium sulfate (40% saturation) increased mean binding content (3.1 ± 1.2 (SD) fmol/mg protein) 15-fold without any change in affinity (10^14 M^-1).

The binding protein sedimented at 8-9S on 10-30% low salt sucrose gradients and 9-10S in the presence of 20 mM molybdate ion. Addition of 0.4 mM KCl shifted the 8S peak to 4S. Binding was specific, saturable, and indicative of a single class of high affinity sites over a concentration range of 0.01-10 mM [H]estradiol. Estradiol produced a dose related inhibition of HM-1 growth in vitro without altering the growth of an additional line (HM-2) which did not bind estrogen. The antiestrogen tamoxifen (10^-7 M) also significantly inhibited HM-1 melanoma growth in vitro, which was reversed by the addition of estradiol (10^-6 M). HM-1 xenografts grew faster in female BALB/c-su/su mice than male mice while there was no sex difference in HM-2 growth. Pharmacological doses of estradiol and the antiestrogen nafoxidine significantly inhibited HM-1 growth without altering tumor incidence or latency.

Our observations suggest that HM-1 cell lines bind estrogens specifically and with high affinity and that hamster melanoma cells positive for this binding protein respond to estrogen.

INTRODUCTION

The response of human melanoma to gonadal steroids remains a controversial issue. Although sex is reported to be prognostically favorable for survival (1-4), malignant melanoma responds poorly to hormonal ablation (5) and variably to additive therapy with estrogens (5), antiestrogens (6-8), or progestins (9-10). This has been suggested to result from the presence or absence of receptors for estrogen (11, 12) although doubts have been raised as to whether these estrogen binding proteins represent functional receptors (13, 14).

The murine (15-18) and hamster (19, 20) melanomas available as models for human melanoma also respond variably to gonadal steroid manipulation in an apparently species and tumor cell dependent manner. Conflicting reports suggest that murine B16 melanoma grows faster (16) or slower (15, 17) in female syngenic C57BL/6J mice than in males, or exhibits no sex difference in growth (18). Castration may increase the rate of B16 melanoma growth in male mice or show no effect (16, 18). Oophorectomy has been reported to either increase the growth rate of B16 melanoma comparable to that in intact or castrated males or exert no effect on this tumor (15-17). Estradiol has also been reported to produce a dose dependent increase in murine B16 growth in male and female mice (16).

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The abbreviations used are: ER, estrogen receptor; DT, doubling time; MEM-E, minimum essential medium-Earle's salts; BSA, bovine serum albumin; PS, protamine sulfate; HAP, hydroxyapatite; DCC, dextran-coated charcoal; TAM, tamoxifen; FBS, fetal bovine serum; DES, diethylstilbestrol; TED (buffer), 10 mM Tris-HCl, 5 mM EDTA-1 mM dithiothreitol, pH 7.4.

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Joel Fortner (Memorial Sloan-Kettering) and subcloned in our laboratory, were maintained in MEM-E supplemented with 16% FBS. These cell lines described are moderately (HM-1) to heavily (HM-2) pigmented. For growth studies, cells were removed with 2 mM EDTA in phosphate buffered saline (10 mM phosphate-0.9% NaCl solution), washed in serum free MEM-E, and 5 x 10^6 cells were inoculated in 12-x 75-mm culture flasks containing MEM-E plus 10% FBS stripped of free steroid with activated charcoal. Steroids and TAM were dissolved in absolute ethanol and brought to final concentration with MEM-E plus 10% charcoal-stripped FBS. The final concentration of ethanol was 0.1%. Control cultures also contained 0.1% ethanol. Growth studies were carried out for 10 days with media changes on days 4, 7, and 9. Cultures were washed with fresh MEM-E (without FBS), cells removed with 2 mM EDTA in phosphate buffered saline, and aliquots counted in a Model ZBI Coulter Counter (Coulter Electronics, Hollywood, FL). Viability was determined using a hemocytometer. Trypan blue exclusion indicated greater than 95% viability in all cases.

Athymic Mice. Mice were held in plastic filter cages on sterile wood shavings within a laminar flow hood at 24°C and constant humidity under a 14-h light/10-h dark lighting regimen with ad libitum access to sterile mouse chow and water. Cells (5 x 10^6) were inoculated s.c. in 0.2 ml MEM-E to the right flank of 4-5-week-old male or female mice (10 mice/group). Tumor incidence and latency were determined by daily inspection and longest tumor diameter determined every other day until the longest tumor diameter reached 1 cm. Tumor volumes were then calculated every other day (33). Estradiol was injected s.c. in 0.1 ml corn oil beginning the day after tumor cell inoculation. Control mice received the corn oil vehicle only. Nafoxidine was dissolved in 10% ethanol saline and injected s.c. Injections continued on alternate days. At necropsy, a portion of the flank tumor was removed for histological examination and the remainder immediately trimmed, rinsed in ice-cold buffer, blotted, cut into small pieces, and frozen in liquid nitrogen for receptor assay. Storage, when necessary, prior to assay was at -80°C.

Cytosol Binding Assay. Tumor specimens were pulverized at liquid nitrogen temperatures and immediately homogenized in TED buffer at a 1:8 dilution (w/v) at 0-4°C with two 10-s bursts of a Polytron P-10 homogenizer (Brinkman Instruments) set at half speed and separated by a 30-s cooling interval. All subsequent procedures were carried out at 0-4°C unless otherwise noted. Homogenates were centrifuged at 800 x g for 10 min. The supernatant solution was aspirated and rescentrifuged at 105,000 x g for 1 h in a titanium 70.1 rotor. Cytosol aliquots (200 µl) were incubated with 0.05-7.0 nM [3H]estradiol in the presence and absence of a 100-fold excess of DES for 16 h. In some experiments cytosols were precipitated with ammonium sulfate to 40% saturation prior to addition of ligand. Protein was determined by the method of Bradford (34) to obviate the increased background melanin adds to the Lowry method of protein assay. Previous reports (29, 31, 35) had suggested that the K_d of melanoma cytosols for estrogen ranged from 0.2 to several nm, depending on species, using DCC (36) separation of bound and free ligand. DCC has been reported to be less effective than PS (37) or HAP (38, 39) in resolving high affinity sites and present an inaccurate estimate of receptor incidence and content in melanoma cytosols (13, 14). Therefore, bound and free [3H]-ligand were separated in cytosols pooled from a heterogenous population of tumor cells by DCC (36), PS (37), and HAP (39) in order to determine whether the lower affinity binding represented an additional binding component or was a function of assay sensitivity.

Tyrosinase Binding Assay. Aliquots (200 µl) of increasing concentrations (0.01-2.0 mg/ml) of soluble mushroom tyrosinase in TED were incubated with 0.5 nm [3H]estradiol for 16 h at 4°C in the presence and absence of a 100-fold excess of DES. In addition, 0.2 mg/ml mushroom tyrosinase (13, 34) was incubated with 0.05-5.0 nM [3H]estradiol at 4°C for 16 h. DES (100-fold excess) was used to correct for nonspecific binding. Results are expressed according to the method of Scatchard (40).

Sucrose Gradients. Sucrose gradients (10-30%) were constructed in TED containing 10% glycerol. Cytosol aliquots (0.5 ml) were incubated for 4 h at 0°C with 10 nm [3H]estradiol in the presence or absence of 0.4 M KCl or 20 mM molybdate ion. Parallel tubes were incubated with a 100-fold excess of DES. Aliquots (250 µl; 8-10 mg/ml protein) were layered on a 1-ml 1,000 g × 10,000 × g, for 16 h in an SW 50.1 swinging bucket rotor. Tube bottoms were pierced and 10-drop (135 µl) fractions collected under constant pressure and counted in 8 ml aqueous scintillation cocktail. All gradients incorporated 4C-labeled BSA into each tube as a 4.6S internal standard. Sedimentation coefficients were estimated from the center of radioactive peaks by the method of Martin and Ames (41).

RESULTS

Cytosol Receptor Characterization. Cytosol protein concentrations in the range of 1-6 mg/ml were routinely used in determining binding affinity and content since this fell within the linear portion of a plot of specifically bound ligand versus protein concentration in HM-1 cells. Maximum specific binding at 0-4°C occurred at 4 h and remained stable for at least 24 h. Initial use of DCC to separate bound from free ligand in

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Fig_1.png}
\caption{Saturation analysis of [3H]estradiol binding to receptor. Values are fmol (f m) bound/200-µl assay aliquot. Saturable binding occurs at about 1 nM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_2.png}
\caption{Specificity of receptor protein for estrogens. Points, mean of triplicate determinations. The deviation about each point (three experiments) is within the symbols. L-Dopa and L-tyrosine appear to exhibit noncompetitive binding. Affinity for DES is ~6-fold estradiol or ethinyl estradiol, ~100-fold theoretical displacement of TAM.}
\end{figure}
Fig. 3. A, sucrose density gradient of [3H]-estradiol binding to UM-1 cytosol in TED buffer in the absence of 20 mM molybdate ion. ▲, 4.6S 14C-labeled BSA marker. ▲, 100-fold; ○, 500-fold; □, 1000-fold excess radioinert estradiol. A similar profile is obtained with radioinert DES. B, [3H]estradiol (E2) binding to HM-1 cytosol in the presence of 20 mM molybdate ion. ▲, 4.6S marker. Radioinert DES is present in 100-fold excess. C, [3H]-estradiol binding (●) to HM-1 cytosol in the presence of 0.4 M KCl. ●, 100-fold excess radioinert DES; ▲, 4.6S region of gradient.

Fig. 4. Scatchard analysis of specific [3H]estradiol bound to 0.1 mg/ml purified mushroom tyrosinase in the presence of a 100-fold excess of radioinert DES. The results are not indicative of specific, high affinity binding.

HM-1 cytosols resulted in a mean binding affinity ($K_d$) of 2.3 nM. Although saturation and Scatchard analysis did not immediately suggest the presence of two distinct binding components with DCC separation (data not shown), analysis of covariance of the slopes ($-1/K_d$) of Scatchard plots ($n = 12$) describing the three methods of separation in identical cytosols revealed significantly different values ($P < 0.05$). When assays which used PS or HAP were combined ($n = 9$) for analysis there was no significant difference in slope. The mean equilibrium $K_d$ from Scatchard analysis in which HAP or PS was used was 0.16 ± 0.09 (SD) nM ($n = 9$). Moreover, in pooled cytosol, the mean $B_{max}$ from experiments in which DCC was used (11.8 ± 1.8 fmol/mg cytosol protein ($n = 3$) differed ($P < 0.05$) from the combined average in which HAP or PS was used (3.2 ± 1.3 fmol/mg cytosol protein; $n = 9$) with a calculated 95% confidence interval of 2.2–4.0 fm/mg cytosol protein. In the presence of PS or HAP the assay system was sensitive and extremely reproducible.

Precipitation of estrogen binding protein with ammonium sulfate to 40% saturation and separation of bound from free ligand with HAP resulted in a 15-fold purification of receptor based on mg protein ($K_d = 4.8 \times 10^{-10}$ M; 43.0 fmol/mg). Saturation analysis showed that although the quantity of non-specific binding relative to total binding remained significant,
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Fig. 5. Dose related effect of estradiol on HM-1 proliferation in vitro. Points, mean of three flasks. SE is within the symbols in all cases. Doubling times in h (days 2–7) are similar for control (O) and 10^{-10} (O), 10^{-9} (A), and 10^{-7} M (W) estradiol, 26.4 h; x, 10^{-8} M estradiol DT, 36 h. Days 7–10, control, 26.4 h, 10^{-7}; 10^{-5} M estradiol, 36 h; 10^{-4} M estradiol, 60 h. *P < 0.05 from respective control point.

Fig. 6. Effects of TAM and estradiol on log phase HM-1 growth in vitro. •, control; •, 10^{-10} M estradiol; x, 10^{-7} M TAM; △, 10^{-5} M TAM; 10^{-4} M estradiol added on day 4. Points, mean of three flasks with SE within each symbol. *P < 0.05.

Fig. 7. Lack of effect of estradiol on HM-2 growth in vitro. •, control; ○, 10^{-6}; △, 10^{-5}; □, 10^{-4} M estradiol. Points, mean of three flasks with SE within symbols.

Fig. 8. Dose related decrease in HM-1 tumor growth in male athymic mice. Estradiol (E2) was injected s.c. on alternate days in corn oil. Points, mean of 10 mice. By day 10 each point is significantly different from control in treatment groups and significantly different between treatments. Tumor diameter rather than volume is plotted because the high dose treatment group never reached 1.0 cm.

high affinity saturable binding was readily and consistently demonstrable over a wide range of ligand concentration even in the absence of ammonium sulfate precipitation when HAP was used to separate bound from free ligand (Fig. 1). Binding in pooled HM-1 cytosols was also indicative of a single class of high affinity receptors with no evidence of cooperation when analyzed by the method of Hill (43) (bH = 1.00; r = 0.98, Kd = 0.44 nM) (43). In contrast, no high affinity binding was present in HM-2 cells using DCC, PS, or HAP to separate bound from free ligand prior to or following precipitation of cytosols with (NH4)2SO4.

Receptor binding was specific for estrogens. Dihydrotestosterone competed slightly (30% maximum inhibition) when pres-
Effect of Estradiol on Melanoma Growth. Continuous exposure of cell line HM-1 to estradiol at concentrations of $10^{-9}$ and $10^{-7}$ M did not decrease log phase growth (DT, 26.4 h) from control (26.4 h) over days 2–7 while $10^{-6}$ M estradiol inhibited growth slightly (36 h). Inhibition was significant over days 7–10 when estradiol inhibited growth at $10^{-9}$ and $10^{-7}$ M (36 h) and $10^{-6}$ M (60 h; $P < 0.05$) (Fig. 5). As there was no significant change in plating efficiency in the presence of estrogen and cells do not reach confluence until day 12 in culture, the effect is apparently on cell proliferation and dependent on concentration and duration of exposure. TAM ($10^{-7}$ M) also significantly decreased the growth of ER positive HM-1 cells after 7 days of exposure (Fig. 6). The growth inhibitory effect of $10^{-7}$ M TAM was reversed on days 9–10 when a physiological concentration ($10^{-9}$ M) estradiol was added on day 4 (Fig. 6). Although TAM produced a significant decrease in growth compared to control over days 7–10 of culture a slight increase in growth in TAM-treated cells on day 10 suggests a differential response of HM-1 cells to TAM. 5α-Dihydrotestosterone ($10^{-9}$–$10^{-6}$ M) had no effect on log growth of HM-1 cells over 10 days of culture (data not shown). Neither estradiol (Fig. 7), TAM, nor 5α-dihydrotestosterone altered the growth of ER negative HM-2 cells on any day of culture when tested at identical concentrations.

Injection of $5 \times 10^5$ HM-1 or HM-2 cells s.c. to male and female athymic mice resulted in a 100% incidence of take with no sex difference in tumor latency (HM-1, 4.1 ± 1.1 days, males; $n = 20$; $4.0 \pm 0.0$, females; $n = 10$; HM-2, 4.3 ± 0.9, males; $n = 8$; 4.2 ± 0.4, females; $n = 10$). Overall, HM-1 growth, however, was significantly faster in female mice (DT, 4.0 days versus 6.1 days; $P < 0.05$) reflecting previous observations in female hamsters (12, 13). This was not observed for ER negative HM-2 cells where growth rates were similar in male and female athymic mice (DT, 6 days). Male athymic mice responded to increasing concentrations of estradiol with a dose related decrease in HM-1 growth (Fig. 8) with no change in tumor latency. Estradiol did not alter the growth of HM-2 cells following transplantation into male athymic mice. The antiestrogenic effect was not restricted to tamoxifen in vitro as nafinoxidine also significantly inhibited HM-1 tumor growth (Fig. 9) following an initial delay but had no effect on HM-2 growth.

**DISCUSSION**

Our results suggest that the growth of a subpopulation of the hamster malignant melanoma MM1 is inhibited by estradiol in a dose dependent manner. Cytosols from these cells (HM-1) bind estradiol specifically and with high affinity. The estrogen binding protein sediments in the 8–9S region of low salt sucrose glycerol gradients (data not shown).
The choice of assay conditions appears critical when characterizing estrogen receptor binding in pigmented cells (13, 14). Affinity for receptor as well as amount bound was a function of the method of separation of bound from free ligand. Binding studies of [3H]estradiol to soluble mushroom tyrosinase have suggested that the radiolabeled by-products of the tyrosinase catalyzed oxidation of [3H]estradiol at the C-1 position are not absorbed by DCC (14). Utilization of HAP and sulfhydryl reducing agents to circumvent the problem of oxidative degradation of estradiol by tyrosinase was also reported to allow quantification of receptor for estrogen in melanoma cytosols (14). Our results agree with this observation and further suggest that partial purification of the cytosol binding protein with ammonium sulfate provides for an additional method to reduce nonspecific binding of [3H]estradiol independent of the level of cellular pigmentation.

The lack of demonstrable high affinity DES suppressible binding of [3H]estradiol to mushroom tyrosinase on Scatchard analysis using DCC or HAP in the present study may stem from the fact that the majority of mammalian tyrosinase is particulate bound and not stabilized to any significant extent by the assay procedure chosen (51). It is not unlikely that separation of bound from free estradiol with DCC may be partially responsible for the variable incidence and generally lower affinity (nm) reported for ER in human melanoma (11–14, 35, 46).

The lack of measurable binding and absence of any effect on the growth of ER negative HM-2 cells suggests that the original MM1 is a clonally heterogeneous tumor, with regard to cellular estrogen binding, whose response is in part determined by the number of receptor positive cells in a particular tumor. This may also represent, in part, the variable response of the generally ER positive albeit heterogeneous murine B16 cell line to hormonal manipulation (15–18). Irrespective of host species, melanomas cellually heterogenous for receptor may, like breast cancer, respond to hormonal manipulation only if the sum total of ER positive cells is sufficient to be reflected as an objective response. The initial delay in inhibiting growth of ER positive HM-1 cells in culture may be due to a secondary estrogen-inducible increase in the production of melanin (21) and its metabolic by-products which are known to inhibit melanoma growth in vitro (52). An absence of any effect in the highly pigmented ER negative HM-2 cell line suggests that this mechanism, if intact, is specific and relegated to ER positive cell lines. Several observations suggest growth inhibition is not via this pathway: (a) when estradiol has been demonstrated to alter cell proliferation in other ER positive cell lines such as the human breast cancer cell line MCF-7, significant changes in growth occur only after 3 days' exposure, if at all (53–55); (b) our results show the effect is present in vivo when melanin by-products would be carried away from these highly vascular lesions; and (c) progestins, rather than estrogens, apparently stimulate melanogenesis at the expense of melanocyte growth (56). It remains to be demonstrated whether the inhibitory effect of estrogens on HM-1 growth is indirect via another mechanism.

Overall host endocrine environment may also dictate tumor response. This would also partially explain the variability of murine melanoma to hormonal manipulation and the increased growth rate of hamster melanoma in female animals. Our observation that xenograft growth in female athymic mice is greater than in males suggests that the phenomenon is independent of species.

The inhibitory effect of higher doses of estradiol also appears independent of the host immune system since estradiol inhibited growth in vitro. Estradiol would be expected to increase rather than inhibit HM-1 growth or at least decrease latency in athymic mice if residual natural-killer cell activity was suppressed in these prepubertal mice (25). Neither of these occurred. An indirect effect of cachexia due to prolonged estradiol administration can also be ruled out as mice did not exhibit any weight loss or other signs of morbidity over the course of the study. The dose related decrease in HM-1 growth and absence of an effect in HM-2 cells in response to estradiol support a hypothesis of estrogen mediating its effect via specific functional receptors.

A similar argument can be made for the inhibitory effect of antiestrogens in ER positive melanoma cells. Tamoxifen has previously been reported to stimulate [3H]thymidine uptake but inhibits the growth of human melanoma in vitro (57) suggesting that TAM may have the potential to inhibit or accelerate the growth of human melanoma. An increase in the SD of cell number after 9–10 days' exposure in the present study suggests that TAM may be selecting out a more “responsive” clone from a heterogenous population of “responsive” and “nonresponsive” ER positive HM1 cells or exerting a partial estrogenic effect at this relatively low concentration of TAM. These observations are similar to those reported for MCF-7 breast cancer cells. MCF-7 breast cancer growth is inhibited in vitro following 6–8 days' exposure to 10−7 M TAM and maximally by 10−6 M TAM (53). This inhibition of cell growth at μM concentrations of TAM is reversed at higher concentrations (10−8–10−7 M) estradiol. As TAM at low doses can act as a partial agonist and even induce receptor for progesterone (53), it is probable that lower doses of estradiol could reverse its growth inhibitory effects. It remains to be determined whether higher concentrations of estradiol will reverse the effect of higher concentrations of TAM in this cell line as it does in others. This phenomenon, if confirmed in additional melanoma cell lines, may help to explain the variable response of patients with ER positive tumors receiving different dosage regimens of antiestrogen for advanced melanoma (6–8).

REFERENCES
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