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Steroid Hormone Receptor Studies in Melanoma Model Systems

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The transplantable B-16 melanotic melanoma carried in syngeneic C57B1/6J female mice and the Syrian hamster melanoma cell line, RPMI 3460, were utilized to determine whether steroid-hormone receptors are present in animal melanomas. In the B-16 melanoma, a cytoplasmic-estrogen receptor is detectable, but there is no evidence for androgen or progestin receptors. Some tumors contain a glucocorticoid-binding macromolecule. Sucrose-density gradient centrifugation of cytosol after incubation with [^{3}H]-estradiol revealed an 8S peak that was suppressed by excess radioinert diethylstilbesterol. Binding varied from 5–35 fmoles per mg cytosol protein. Scatchard analysis of [^{3}H]-estradiol binding in cytosol yielded a single class of high-affinity binding sites; the dissociation constant is 6×10^{-10} M. The receptor molecule is shown to be estrogen-specific by ligand competition assays. In contrast to B-16 melanoma, no estrogen, androgen, or progestin receptor can be found in the Syrian hamster melanoma cell line. However, a substantial level of specific binding is observed using [^{3}H]-dexamethasone. Sucrose-gradient centrifugation of cytosol from this cell line after incubation with [^{3}H]-dexamethasone revealed a 7S peak that was suppressed by excess radioinert dexamethasone. Scatchard analysis indicated a single class of high-affinity sites with a dissociation constant of 2×10^{-9} M. Binding levels from 70–610 fmoles per mg cytosol protein were observed. The Syrian hamster melanoma cells also exhibit a biological response to glucocorticoids: Dexamethasone causes both an inhibition of growth and a decrease in final-cell density in these cells.

Key words: estrogen receptor, glucocorticoid receptor, estradiol, diethylstilbestrol, dexamethasone, B-16 mouse melanoma, Syrian hamster melanoma

Human melanoma exhibits a low response rate to chemotherapeutic drugs, and novel or alternative forms of therapy are vitally needed for this disease. The possible hormonal dependence of human malignant melanoma has been debated for a number of years. Several observations suggest an influence of the hormonal environment on the course

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of the disease. These observations include the relatively low incidence of melanoma prior to puberty, the higher incidence of melanoma during the childbearing years in women, the significantly better survival of females than males with malignant melanoma, and the favorable response in a small number of male and female patients to alteration in their endocrine environment [1, 2].

Recently it has been demonstrated that human melanomas contain steroid-hormone receptors [3–7] and that the growth of human melanoma cell lines is altered by steroid hormones [8, 9]. Characterization of steroid-hormone receptors and identification of biological activities of steroid hormones in animal melanoma model systems may provide a significant approach toward understanding hormonal effects in human melanoma. With this objective in mind, we undertook the study of endocrine influences and steroid-hormone receptors in both the B-16 melanotic melanoma carried in syngeneic C57B1/6J female mice and the Syrian hamster melanoma cell line, RPMI 3460. In this report, we detail our initial results on receptor characterization in these two systems. Preliminary reports of this work have been published [10–13].

MATERIALS AND METHODS

Materials

[2,4,6,7-^{3}H]-estradiol-17β (96 Ci/mmole), [6,7-^{3}H]-R1881 (methyltrienolone, 17β-hydroxyl-17α-methyl-estra-4,9,11-triene-3-one, 87 Ci/mmole), [6,7-^{3}H]-R5020 (17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione, 56.5 Ci/mmole), radioinert R5020 and R1881, and [6,7-^{3}H]-dexamethasone (9α-fluoro-16α-methyl-11β, 17,21-trihydroxy-1,4-pregnadiene-3,20-dione, 33 Ci/mmole) were obtained from New England Nuclear (Boston , MA). Radioinert estradiol-17β diethylstilbestrol, dexamethasone, triamcinolone acetonide, cortisol, 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one), and testosterone were obtained from Sigma Chemical (St. Louis, MO). Estradiol-17α, estrone and estriol were purchased from Steraloids (Wilton, NH). Progesterone was supplied by Calbiochem (La Jolla, CA). R2858 (moxestrol), 11β-methoxy-17-ethynyl-1,3,5 (10)-estratriene-3, 17β-diol) was a generous gift from Dr J-P. Raynaud (Roussel-Uclaf, Romainville, France). Purity of the radiolabeled steroids was checked periodically by thin-layer chromatography. The steroids were used if found to be greater than 95% pure.

Reagent grade Trisma base, dithiothreitol, and sucrose (grade 1) were obtained from Sigma Chemical. Ethylenediaminetetraacetic acid (EDTA) was purchased from J. T. Baker (Phillipsburg, NJ). Radioimmunoassay-grade charcoal was purchased from Schwartz-Mann (Orangeburg, NY). Dextran T-70 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents were of analytical reagent grade.

Tumors and Tissue: B-16 Melanotic Melanoma

B-16 melanotic melanoma-bearing C57B1/6J mice were obtained from Southern Research Institute (Birmingham, Alabama). The tumor line was stored in liquid nitrogen and maintained by serial subcutaneous transplantation into the flanks of C57B1/6J female mice. Normal C57B1/6J female mice were supplied by Mr. Clarence Reeder of the Division of Cancer Treatment, National Cancer Institute.

Tumors became palpable after about 8–10 days and were removed from the animals for study 11–14 days after transplantation. The tissue was stored in liquid nitrogen until receptor studies were performed. Standard hematoxylin- and eosin-stained preparations were made of representative sections from the tumors for histopathological identification.

Syrian Hamster Melanoma

The Syrian hamster melanoma cell line, RPMI 3460, is a highly tumorigenic, pigmented cell line originally established in cell culture from a transplantable Syrian hamster tumor [14]. The 3460 cell line is maintained in monolayer cultures in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (growth medium). For determinations of hormone receptor, 2×10^6 of the 3460 cells were inoculated per 150 mm^2 tissue-culture dish and grown for seven days. Cells were harvested by trypsinization, washed two times with Ca^{++}-Mg^{++}-free phosphate-buffered saline (PBS) and one time with PBS containing 1.5 mM EDTA and 0.5 mM dithioerythritol. Cells were stored in liquid nitrogen until used for receptor studies.

Effect of Glucocorticoids on Growth of Syrian Hamster Melanoma Cells

To test the effect of dexamethasone on the growth of the 3460 cell line, 10^5 cells were inoculated per 60 mm^2 tissue-culture dish. After 24 hours, dexamethasone at concentrations from 10^{-6} M to 10^{-9} M was added to different dishes. Dexamethasone was dissolved in water and diluted to the appropriate concentration with growth medium. Every two days, cells were collected by trypsinization and counted using a hematocytometer. Cell counts represent the average of determinations from two separate dishes and are accurate to ± 10%.

Preparation of Cytosol

Frozen pieces of B-16 melanoma, mouse skin, or the 3460 melanoma cells were pulverized in a Thermo Vac tissue pulverizer (Thermo Vac Industries, Copiague, NY) at liquid-nitrogen temperature. Cytosol was prepared as previously described [15], except that stripping of endogenous steroids was omitted.

Dextran-Coated Charcoal Assay

Cytosol was incubated with various quantities of [^{3}H]-steroid for 20 hours at 0°C using the dextran-charcoal procedure, as previously described [16]. Scatchard plots [17] of total binding were constructed after determining the amount of bound and free steroid. These plots were corrected for nonspecific binding as suggested by Chamness and McGuire [18], and dissociation constants (K_d) and total binding sites were determined from plots of specifically bound [^{3}H]-steroid.

The dextran-charcoal procedure [15] also was used to study the effect of cytosol-protein concentration on binding of [^{3}H]-steroid and to measure the time course of [^{3}H]-steroid uptake by cytosol.

Ligand specificity studies were performed by incubating 5 nM [^{3}H]-estradiol in the presence or absence of various concentrations of radioinert steroid for 16–20 hours at 0°C. Specific binding was determined by the dextran-charcoal procedure, as previously described [15]. Binding in the presence of competing steroids was expressed as percent of specific binding.

Sucrose-Density Gradient Centrifugation

Cytosol was incubated with steroid for 3 or 20 hours at 0°C and analyzed by overnight sucrose-density gradient centrifugation [19] in the SW60Ti rotor. Occasionally, where noted, samples were centrifuged for 3 hours in the VTi-65 vertical tube rotor.

Steroid Binding by Mouse Plasma and Skin

In view of the possible occurrence of binding compounds in mouse blood or skin that could complicate determinations of steroid hormone receptors in melanoma cytosol,

[^{3}H]-steroid binding by C57B1/6J mouse plasma and skin was investigated. Mouse blood was collected by cardiac puncture, and the plasma was treated with four volumes of dextran-charcoal for 10 minutes at 0°C with intermittent shaking to remove free steroids. After centrifugation, the diluted plasma was incubated with [^{3}H]-steroid for 3 hours at 0°C with or without 100-fold molar excess radioinert competitor. The incubation was terminated by treatment with dextran-charcoal to remove excess steroid, and 200 μl aliquots were analyzed by sucrose-density gradient centrifugation. Mouse-skin cytosol also was analyzed by sucrose-density gradient centrifugation after incubation at 0°C for 3 hours with [^{3}H]-steroid, either alone or in the presence of 100-fold molar excess radioinert competitor.

Protein Determination

Protein concentrations of cytosol preparations were determined by the method of Lowry et al [20] using human serum albumin and human γ-globulin as standards. B-16 melanoma cytosol protein concentrations ranged from 8–20 mg/ml; Syrian hamster melanoma cytosol protein concentrations varied from 3–14 mg/ml.

RESULTS

Histopathology of B-16 Melanoma

Histopathology of B-16 melanoma at harvest gave the following general pattern: Tumors consisted of a uniform homogeneous population of large oval, polygonal, or elongated cells with moderate to large amounts of cytoplasm. In many cells the pigment granules are small and few in number, but in a few cells numerous large pigment granules completely fill the entire cytoplasm. Mitotic figures are noted with moderate frequency. Scattered areas of cell necrosis are present. Overall, more than 90% of the tumor is viable. A small amount of fibrovascular stroma is noted within the tumor.

Steroid Hormone Receptor Characterization of B-16 Melanotic Melanoma: Estrogen Receptor

Titration of cytosol from B-16 melanotic melanoma with increasing concentrations of [^{3}H]-estradiol indicated that an estrogen-binding protein was present that was saturated at 1–2 nM [^{3}H]-estradiol. Scatchard analysis of this data [17] – when corrected for nonspecific binding by the method of Chamness and McGuire [18] – produced a straight line, suggesting a single class of high-affinity estradiol binding sites (Fig. 1). The estrogen binding protein exhibited an average K_d of 5.5×10^{-10} M (SD = 2.1×10^{-10} M for five determinations). Total binding ranged from 3–18 fmoles per mg cytosol protein (fmoles/mg). The average binding (five determinations) is 10.1 fmoles/mg. The K_d is in the range expected for an estrogen receptor, based on findings in uterine tissue [21] and breast tumor tissue [22].

To further characterize estrogen binding, the effect of cytosol-protein concentration on steroid binding was evaluated, using the dextran-charcoal method. Specific binding increased linearly with cytosol-protein concentration (cytosol dilution) from 3–15 mg/ml. Thus, assays for steroid-receptor levels are reliable within this range. The time course of binding of [^{3}H]-estradiol was also analyzed, and it was found that binding was maximal after two hours of incubation at 0°C. After saturation was reached, the amount of specifically-bound [^{3}H]-estradiol remained stable for at least 28 hours at 0°C. It is possible, therefore, to measure [^{3}H]-estradiol binding by either three-hour or overnight incubation of cytosol at 0°C.

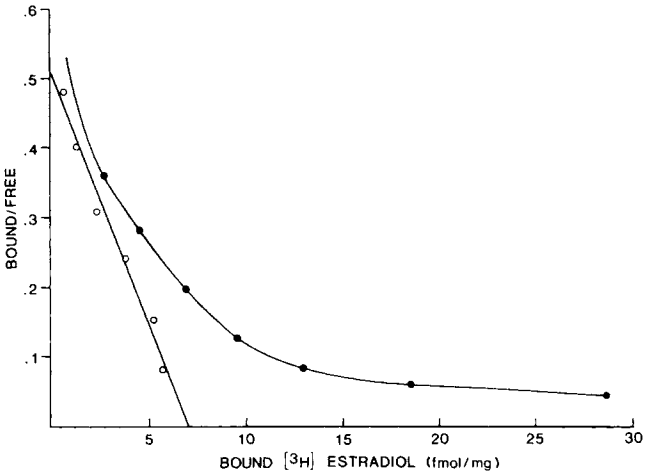

Fig. 1. Scatchard plot of estrogen binding in B-16 melanoma. Cytosol was incubated for 20 hours at 0°C with increasing concentrations of [^{3}H]-estradiol from 0.02–10.3 nM. The dextran-charcoal competitive-binding assay was used as described in "Materials and Methods" to measure total binding (•–•). Correction for nonsuppressible binding was accomplished by the method of Chamness and McGuire [18]. Scatchard analysis of specifically-bound steroid (○–○) indicated a K_d of 2.1×10^{-10} M and a binding capacity of 7.1 fmoles/mg. Protein concentration was 15.5 mg/ml.

Some molecular characteristics of the estrogen receptor were also investigated. B-16 melanoma cytosol was incubated with 6 nM [^{3}H]-estradiol for 3 (or 20) hours at 0°C and was analyzed by overnight sucrose-density gradient centrifugation in low-salt buffer. Binding of [^{3}H]-estradiol to a macromolecular component with a sedimentation coefficient of 7.8S (average of eight determinations, range from 7.5–8.5S) was observed. This binding was completely suppressed by 100-fold molar excess radioinert diethylstilbestrol. In most samples, there also was some suppressible binding in the 4–5S region (Fig. 2). The finding of suppressible 8S binding in low-salt gradients for the estrogen-binding macromolecule is in agreement with findings in other tissues [22, 23] and provides further support that the estrogen-binding protein in B-16 melanoma is an estrogen receptor. Quantitation of estrogen binding by sucrose-density gradient centrifugation of seven different B-16 melanoma cytosol preparations revealed a range of 3.5–36.1 fmoles/mg (average = 16.9 fmoles/mg; SD = 11.1 fmoles/mg).

To study ligand specificity of the estrogen-binding macromolecule in B-16 melanoma, cytosol was incubated with 5 nM [^{3}H]-estradiol in the presence or absence of various concentrations of radioinert competitors and specific binding was measured by the dextran-charcoal procedure (Fig. 3). Estradiol-17β was an effective inhibitor of [^{3}H]-estradiol binding; R2858 was not quite as effective. Androgens (testosterone, R1881, and 5α-dihydrotestosterone), progestin (R5020 and progesterone), and glucocorticoids (dexamethasone, cortisol, and triamcinolone acetonide) were ineffective competitors for [^{3}H]-estradiol binding (Fig. 3). Different synthetic and natural estrogens exhibited varying effectiveness as competitors of [^{3}H]-estradiol binding; the concentrations for 50% inhibition (I_{50}) were approximately 8×10^{-8} M, 2×10^{-7}, 1×10^{-7} M and 3×10^{-7} M, for R2858, estradiol-17α, estriol, and estrone, respectively. For comparison, the I_{50} for estradiol-17β was 7×10^{-9} M. Inhibition curves for testosterone, cortisol, estradiol-17α, estriol, and estrone are not presented in Figure 3.

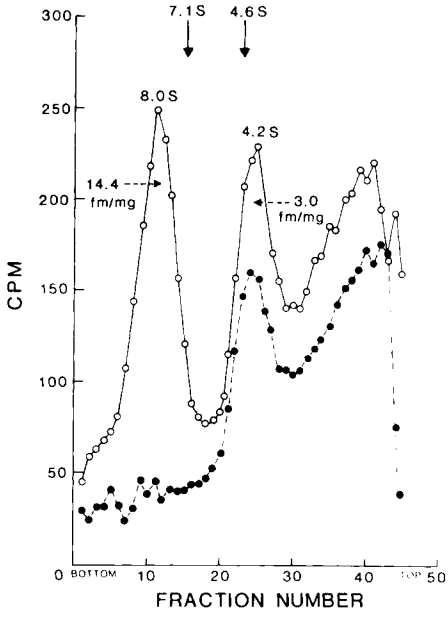

Fig. 2. Representative sucrose density gradient-centrifugation profile in 0.01 M Tris buffer (see Materials and Methods) of B-16 melanoma cytosol after incubation with 6 nM [^{3}H]-estradiol alone (○–○) or with 600 nM radioinerrt estradiol (●–●). Binding profiles were identical when 600 nM radioinert diethylstilbestrol was utilized in place of radioinert estradiol. Protein concentration was 11.0 mg/ml. Vertical arrows at 7.1S and 4.6S indicate the positions of the marker proteins, human γ-globulin and human serum albumin, respectively. Suppressible binding at 8.0S is 14.4 fmoles/mg and 3.0 fmoles/mg in the 4–5S region.

Partial purification of the estrogen-binding macromolecule was achieved by bringing cytosol to 35% saturation with solid ammonium sulfate at 0°C. The precipitate was then dissolved in cytosol buffer. Scatchard analysis showed that essentially 100% of the receptor had been recovered and that it had a K_d of 1.3×10^{-10} M. This procedure resulted in about five-fold purification of the estrogen receptor. In spite of the fact that ammonium sulfate precipitation was performed in the absence of bound steroid, there was virtually no loss in binding activity. Thus, the estrogen-binding macromolecule in B-16 melanoma behaves like other receptors, both with respect to its precipitability by low concentrations of ammonium sulfate and in terms of steroid affinity of the partially purified receptor [24].

Other Steroid Hormone Receptors in B-16 Melanoma

Cytosol from the B-16 melanoma was analyzed by sucrose-density gradient centrifugation with the vertical-tube rotor for the presence of androgen and progestin receptors. Extracts were incubated with [^{3}H]-steroid in the presence or absence of 100-fold molar excess radioinert competitor. In the assay for progestin receptor, 100-fold molar excess radioinert dexamethasone was included to block binding of [^{3}H]-R5020 to the glucocorticoid receptor. There was no suppressible 8S binding of androgen using 20 nM [^{3}H]-R1881 or of progestin using 6 nM [^{3}H]-R5020. However, occasionally there was a small amount of suppressible binding of [^{3}H]-R1881 or [^{3}H]-R5020 in the 4–5S region. When analyzing cytosol for the presence of a glucocorticoid receptor using 20 nM [^{3}H]-dexamethasone, sucrose-density gradient centrifugation with the vertical-tube rotor produced

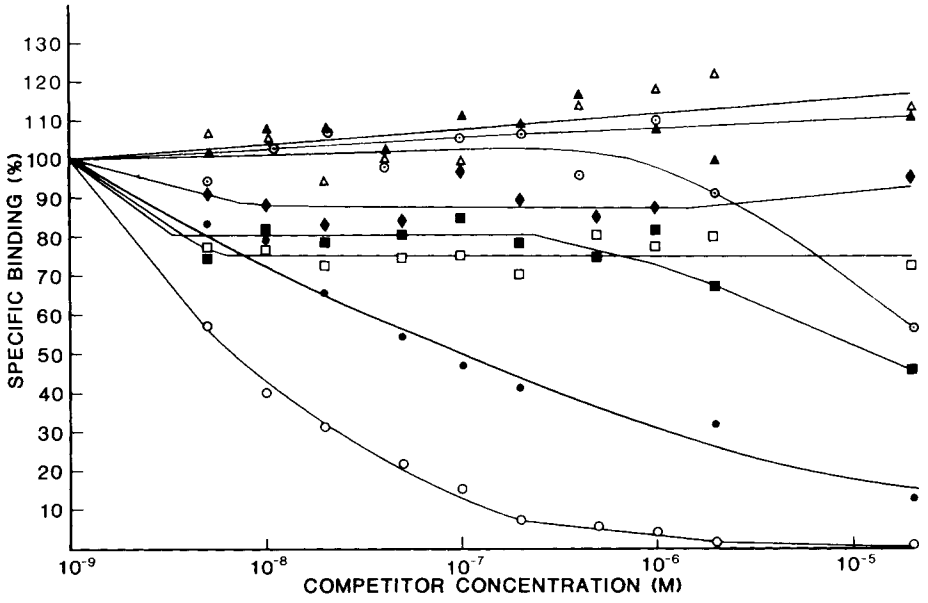

Fig. 3. Ligand specificity studies for the estrogen receptor in B-16 melanoma cytosol. 5 nM [^{3}H]-estradiol was incubated at 0°C for 20 hours in the presence or absence of varying concentrations of competing steroids. The dextran-charcoal procedure was used, and results are expressed as % inhibition of specific binding. The 100% value was determined from the difference in binding of [^{3}H]-estradiol alone or in the presence of 500 nM radioinert estradiol. Protein concentration varied in the different experiments from 10–20 mg/ml. The following competing steroids were used: ▲–▲ dexamethasone; ●–●, R2858; ○–○, estradiol-17β; Θ–Θ, R1881; ♦–♦, R5020; ■–■, triamcinolone acetonide; □–□, progesterone; and Δ–Δ, 5α-dihydrotestosterone.

variable results, depending on the tumor. In some tumors, there was no suppressible 8S binding of [^{3}H]-dexamethasone. However, other tumors contained suppressible binding of [^{3}H]-dexamethasone in both the 8S and 4–5S regions. In one tumor, cytosol binding of dexamethasone was 10 fmoles/mg in the 8S region and 47 fmoles/mg in the 4–5S region (Fig. 4a). By comparison, cytosol binding of [^{3}H]-estradiol was 7.3 fmoles/mg in the 8S region with no suppressible binding in the 4–5S region (Fig. 4b).

Steroid-Hormone Binding in C57B1/6J Mouse Plasma and Skin

Since B-16 melanoma tissue obtained for receptor studies is vascularized, there is a significant contamination of cytosol by plasma proteins. To rule out the possibility that binding observed in cytosol is caused by blood binding components, steroid binding in plasma (protein concentration 5.7 mg/ml) from C57B1/6J mice was investigated by sucrose-density gradient centrifugation using the vertical-tube rotor. There was no binding suppressible by 100-fold molar excess radioinert steroid in the 8S or 4–5S regions using either 6 nM [^{3}H]-estradiol or 20 nM [^{3}H]-dexamethasone.

In separate experiments, cytosol (protein concentration 2.3 mg/ml) prepared from mouse skin was also analyzed for estrogen and glucocorticoid binding by sucrose-density gradient centrifugation. By this criterion, no suppressible binding of either [^{3}H]-estradiol (6 nM) or [^{3}H]-dexamethasone (20 nM) was observed. These experiments indicate that steroid binding by B-16 melanoma cytosol is due to cytoplasmic receptors and not to suppressible binding components from blood or skin.

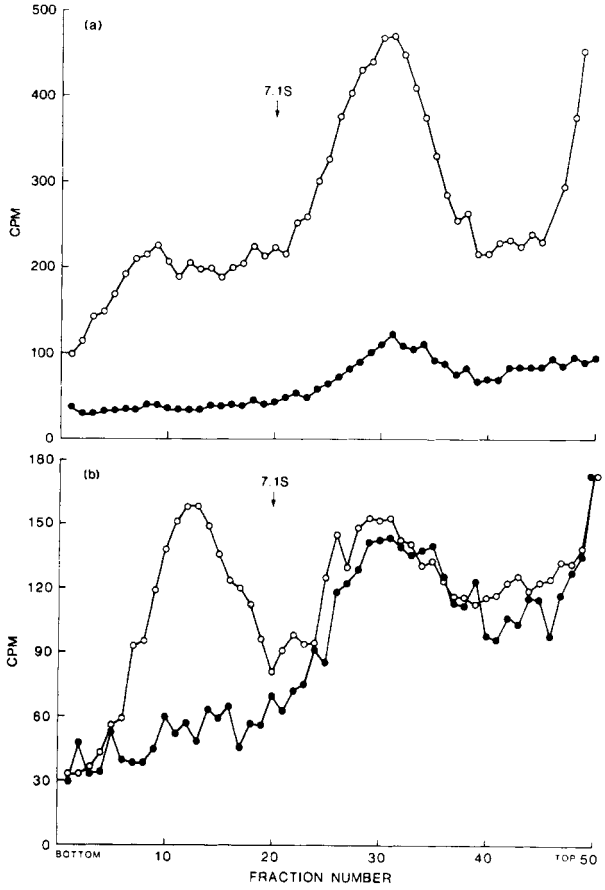

Fig. 4. Representative sucrose-density gradient-centrifugation profile in 0.01 M Tris buffer (see Materials and Methods) of estrogen and glucocorticoid binding in B-16 melanoma, using the VTi-65 vertical tube rotor. The vertical arrow at 7.1S indicates the position of human γ-globulin. (a) 20 nM [^{3}H]-dexamethasone alone (○–○) or with 2 μM radioinert dexamethasone (●–●). Protein concentration was 15.9 mg/ml. The level of suppressible glucocorticoid binding was somewhat variable. However, in this experiment, there were 10 fmoles/mg in the 8S region and about 47 fmoles/mg in the 4–5S. region. (b) 6 nM [^{3}H]-estradiol alone (○–○) or with 600 nM diethylstilbestrol (●–●). Protein concentration was 8.1 mg/ml. Suppressible binding in the 8S region is 7.3 fmoles/mg.

Steroid-Hormone Receptors in Syrian Hamster Melanoma Cell Line RPMI 3460: Glucocorticoid Receptor

The Syrian hamster melanoma cell line, RPMI 3460, was also investigated for steroid binding proteins. Cytosol prepared from 3460 cells was incubated with increasing concentrations of [^{3}H]-dexamethasone from 0.1 to 55 nM for 20 hours at 0°C, and binding was analyzed by the dextran-charcoal procedure. Glucocorticoid-binding sites were saturated between 10–16 nM [^{3}H]-dexamethasone. Scatchard analysis [17] yielded a linear plot, suggesting a single class of binding sites with a K_d of 2.9×10^{-9} M ± 0.8 (average of two determinations, correlation coefficient –0.99). Total binding was 533 ± 120 fmoles/mg (average of two determinations). Correction for nonsuppressible binding [18] was not necessary, since the Scatchard plot of total [^{3}H]-dexamethasone binding was linear at all concentrations tested.

To characterize some molecular properties of the glucocorticoid-binding molecule, sucrose-density gradient centrifugation was performed after incubation of 3460 cytosol

with 20 nM [^{3}H]-dexamethasone for 3 hours at 0°C (Fig. 5). Specific macromolecular binding of [^{3}H]-dexamethasone in low-ionic-strength sucrose gradients had a sedimentation coefficient of 7.0S (average of five determinations, range 6.7–7.8S; SD = 0.5S). Binding in the 7S region was completely suppressed by 100-fold molar excess radioinert dexamethasone. Suppressible binding in the 7S region varied from 70–340 fmoles/mg in different cytosol preparations (average of five determinations = 178 fmoles/mg). The greatest quantity of binding observed, 340 fmoles/mg, occurred after 20 hours of incubation with 50 nM [^{3}H]-dexamethasone.

All of the experimental evidence accumulated suggests that the macromolecular [^{3}H]-dexamethasone-binding protein in 3460 cytosol is a glucocorticoid receptor. Its steroid affinity and sedimentation properties are similar to those of the glucocorticoid receptors found in other tissues such as hepatoma-tissue culture cells [25], leukemia [26], and mammary tumors [27]. Additionally, ligand specificity studies show that the binding protein behaves as a glucocorticoid receptor. Androgens and estrogens have very low affinity for the receptor, progestins and mineralocorticoids have low affinity, and natural glucocorticoids have intermediate affinity. The synthetic glucocorticoids – dexamethasone and triamcinolone acetonide – exhibit very high binding affinity (E. Hawkins, D. Horn, and F. Markland, Cancer Research, in press).

Other Steroid-Hormone Receptors in 3460 Cells

The presence of other steroid-binding molecules in cytosol from 3460 cells was investigated by sucrose-density gradient centrifugation. Binding of androgen, estrogen, and progestin was evaluated after three hours incubation of 3460 cytosol at 0°C with 20 nM [^{3}H]-R1881, 6nM [^{3}H]-estradiol, or 6 nM [^{3}H]-R5020, respectively, in the presence or absence of 100-fold molar excess radioinert competitor. In the assay for progestin binding, 100-fold molar excess radioinert dexamethasone was added to block possible [^{3}H]-R5020 binding to the glucocorticoid receptor. There was no detectable 7–8S sup-

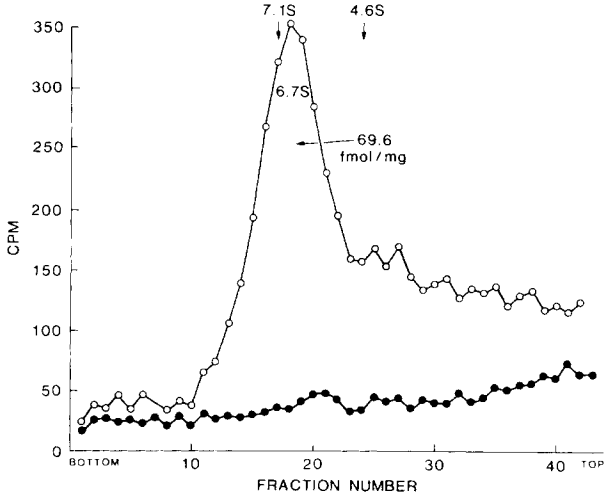

Fig. 5. Representative sucrose-density gradient-centrifugation profile in 0.01 M Tris buffer (see Materials and Methods) of Syrian hamster malanoma cell line RPMI 3460 cytosol after incubation with 20 nM [^{3}H]-dexamethasone, either alone (○–○) or with 2 μM radioinert dexamethasone (●–●). Vertical arrows at 7.1S and 4.6S indicate the position of the marker proteins, human γ-globulin and human serum albumin, respectively. Suppressible binding at 6.7S is 69.6 fmoles/mg. There is additional binding in the 4–5S region, but this is not included in the total for quantitation. Protein concentration was 5.8 mg/ml.

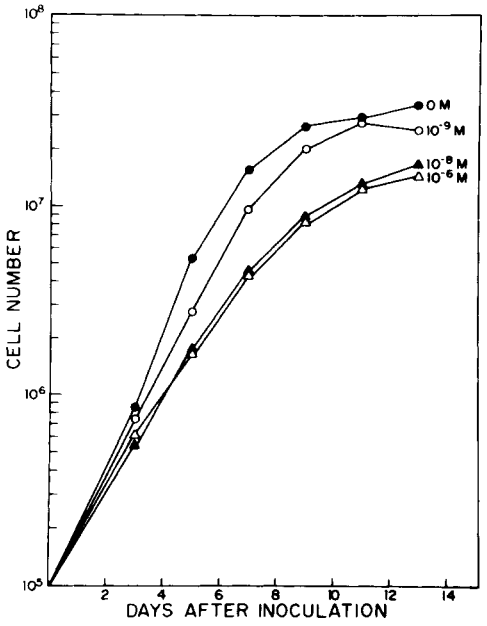

Fig. 6. Growth of 3460 Syrian hamster melanoma cells in growth medium (●–●), in growth medium containing 10^{-9} M dexamethasone (○–○), 10^{-8} M dexamethasone (▲–▲), or 10^{-6} M dexamethasone (Δ–Δ). Cells were treated as described in Materials and Methods.

pressible binding of androgen, estrogen, or progestin. By comparison, there was 260 fmoles/mg suppressible binding of [^{3}H]-dexamethasone in the 7S region of a gradient run with the same tumor cytosol.

Effect of Glucocorticoids on Growth of Syrian Hamster Melanoma Cell Line RPMI 3460

To determine whether 3460 cells exhibit a growth response to glucocorticoids, the cells were grown in the presence and absence of various concentrations of dexamethasone. As shown in Figure 6, the presence of dexamethasone causes an inhibition of growth, and a decrease in final-cell density in the 3460 cells. Cells grown in the absence of dexamethasone have a population doubling time of approximately 18 hours, whereas cells grown in the presence of dexamethasone concentrations of 10^{-8} M and above had population doubling times of over 30 hours. An intermediate-population doubling time of 25 hours was observed when 3460 cells were grown in the presence of 10^{-9} M dexamethasone. As indicated in Figure 6, the final-cell density of 3460 cells grown in the absence of dexamethasone is approximately two-fold greater than the final-cell density of the cells grown in the presence of concentrations of dexamethasone above 10^{-8} M.

DISCUSSION

In this report we provide evidence that the B-16 melanotic melanoma carried in C57B1/6J female mice contains an estrogen-binding protein that satisfies the physicochemical and biochemical criteria for classification as a steroid hormone receptor. Although we have not shown that the estrogen receptor is biologically active, others have demonstrated recently that B-16 melanoma grown subcutaneously in C57B1/6J mice is responsive to estrogens [28]. These workers [28] showed that, not only did exogenous

estrogens have a dose-dependent enhancing effect on tumor growth and rate of pulmonary metastasis, but also that the antiestrogen, nafoxidine, impaired tumor growth. Oophorectomy had no significant effect on tumor growth. These results provide convincing evidence, when taken with our findings, that there is a functional estrogen receptor in B-16 melanoma. Interestingly, it has been reported independently that a highly malignant variant of B-16 melanoma grows more slowly in female than in male syngeneic C57B1/6J mice [29]. However, after oophorectomy, the growth rate is similar in females and normal or castrated males, suggesting that female sex hormones suppress the growth of this tumor.

There does not appear to be suppressible 8S binding of androgens or progestins by B-16 melanoma cytosol. However, in some tumors, there appears to be variable amounts of a suppressible [^{3}H]-dexamethasone-binding macromolecule sedimenting at 7–8S. Others also have reported preliminarily on the presence of a dexamethasone-binding protein in B-16 melanoma and the inhibition of tumor growth by glucocorticoids [30].

The Syrian hamster cell line, RPMI 3460, was devoid of estrogen, androgen, and progestin receptors. However, substantial amounts of a suppressible [^{3}H]-dexamethasone-binding macromolecule were present that behaved like a glucocorticoid receptor. Physiological concentrations of dexamethasone cause inhibition of growth and a decrease in final-cell density of the 3460 cells. Other glucocorticoids such as triamcinolone acetonide and hydrocortisone also have been shown to inhibit the growth of 3460 cells and progesterone, which acts as an antiglucocorticoid in other systems [31], reverses the inhibition of growth by dexamethasone in these cells (D. Horn, in preparation). The results indicate that, in addition to having a glucocorticoid receptor, 3460 cells also exhibit a biological response to glucocorticoids. Thus, the 3460 Syrian hamster melanoma cells can be considered glucocorticoid responsive. Further work on the characterization of the glucocorticoid receptor from 3460 cells and its nuclear translocation are in progress.

Additional studies in other melanoma model systems provide more evidence for the role of the endocrine environment in the development and growth of melanoma and for the involvement of steroid-hormone receptors in these processes. Thus, several investigators have reported on the presence of steroid-hormone receptors in hamster melanoma [32, 33]. Estrogen has been shown to stimulate growth of hamster melanoma, and it has also been reported that these tumors contain an estrogen receptor [32]. Adrenalectomy appears to decrease growth of MMI hamster melanoma, and replacement adrenocorticoids increase tumor growth. The growth responses apparently were correlated with the presence of a glucocorticoid receptor in the MMI melanoma [33].

The significance of work on steroid-hormone receptors in animal melanoma to therapy of the human disease remains to be determined. However, an increasing body of evidence suggests that, at least in some instances, melanoma indeed may be an endocrine-responsive disease. Findings in animal model systems may provide sufficient rationale for the development of effective forms of hormonal therapy in that subset of melanoma patients with demonstrable hormone receptors. The situation may be analogous to the utilization of steroid-hormone receptors in decision making for therapeutic approaches in breast cancer [34, 35].

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REFERENCES

1. Bodenham DC, Hale B: In Stoll BA (ed): "Endocrine Therapy in Malignant Disease." Philadelphia: Saunders, 1972, pp 377–383.
2. Sadof L, Winkley J, Tyson S: Oncology 27:244, 1973.
3. Fisher RI, Neifeld JP, Lippman ME: Lancet 4:337, 1976.
4. Neifeld JP, Lippman ME, Fisher RI: Surg Forum 27:108, 1976.
5. Fondo EY, Mendex-Botet CJ, Schwartz MK: Clin Chem 22:1174, 1976.
6. Friedman MA, Seematter R, Hoffan PG: Clin Res 26:435A, 1978.
7. Chaudhuri PK, Walker MJ, Das Gupta TK, Beattie CW: Endocrine Society 61st Annual Meeting: 148:1979.
8. Fuller BB, Meyskens FL: Proc Am Assoc Cancer Res 20:93, 1979.
9. Beattie CW, Chaudhuri PK, Walker MJ, Das Gupta TK: Proc Am Assoc Cancer Res 20:130, 1979.
10. Markland FS, Varven JC, Khwaja TA: Endocrine Society 60th Annual Meeting: 304, 1978.
11. Markland FS, Horn D: J Cell Biol 75:194a, 1977.
12. Markland FS, Horn D: J Supramol Struct 3 (Suppl):225, 1979.
13. Horn D, Schmidhauser TJ: J Cell Biol 79:193a, 1978.
14. Moore G: Experimental Cell Res 36:422, 1964.
15. Markland FS, Lee L: J Steroid Biochem 10:13, 1979.
16. Markland FS, Chopp RT, Cosgrove MD, Howard EB: Cancer Res 38:2818, 1978.
17. Scatchard G: Ann NY Acad SCI USA 51:660, 1949.
18. Chamness GC, McGuire WL: Steroids 26:538, 1975.
19. Markland FS, Chopp RT, Cosgrove MD, Howard EB: J Supramol Struct 9:509, 1978.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
21. Toft D, Shyamala G, Gorski J: Proc Natl Acad Sci USA 57:1740, 1967.
22. McGuire WL, Chamness GC, Costlow ME, Shepherd RE: In McKerns KW (ed): "Hormones and Cancer." New York: Academic Press, 1974, pp 75–101.
23. Jensen EV, DeSombre ER: Annu Rev Biochem 41:203, 1972.
24. Clark JH, Peck EJ, Schrader WT, O'Malley BW: In Busch H (ed): "Methods in Cancer Research." New York: Academic Press, 1976, vol 12, pp 367–417.
25. Lippman ME: Hormone-receptor interaction: Molecular aspects. In Levey GS (ed): "Modern Pharmacology Toxicology Series." New York: Marcel Dekker, 1976, vol 9, pp 221–242.
26. Schmidt TJ, Thompson EB: Endocrine control in neoplasia. In Sharma RK, Criss WE (eds): "Progress in Cancer Research and Therapy." New York: Raven Press, vol 9, 1978, pp 263–290.
27. Goral JE, Wittliff JL: Biochemistry 14:2944, 1975.
28. Lopez RE, Bhakoo H, Paolini NS, Rosen F, Holyoke ED, Goldrosen MH. Surg Forum 29:153, 1978.
29. Proctor JW, Auclair BG, Stokowski L: J Natl Cancer Inst 57:1197, 1976.
30. Bhakoo HS, DiCioccio RA, Rosen F: Proc Am Assoc Cancer Res 20:201, 1979.
31. Rousseau GG, Baxter JD, Higgins SJ, Tomkins GM: J Mol Biol 79:539, 1973.
32. Synder J, Das Gupta TK: Fed Proc 36:349, 1977.
33. Stanberry LR, Lindsey WF: Endocrine Society 61st Annual Meeting: 151, 1979.
34. DeSombre ER, Greene GL, Jensen EV: Hormones, receptors, and breast cancer. In McGuire WL (ed): "Progress in Cancer Research and Therapy." New York: Raven Press, vol 10, 1978, pp 1–14.
35. McGuire WL, Horwitz KB: Hormones, receptors and breast cancer. In McGuire WL (ed): "Progress in Cancer Research and Therapy." New York: Raven Press, vol 10, 1978, pp 31–42.