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Initial Characterisation of Oestrogen Receptors in Canine Mammary Tumour Lines Maintained in Nude Mice

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Abstract—*Proteins binding oestradiol with high affinity and low capacity have been characterised and quantitated in 4 newly established canine mammary tumour lines, MS306, V5500, F5010 and PD6014, maintained in nude mice. These receptor proteins, specific for oestrogens, were found in all the tumour lines, both in initial implants and all subsequent passages. Receptors had equilibrium dissociation constants for oestradiol in the range of* 33–210 pM, *and sedimentation coefficients of* 4 S *and* 8 S.

INTRODUCTION

OESTROGENS have a controlling role in the growth and development of the normal and neoplastic mammary gland [1]. Hormone-dependent mammary tumours have been demonstrated in the rat [2–4], mouse [5, 6] and dog [7–11]. Attempts to establish transplantable mammary tumour lines in nude mice [12–16] have met generally with little success except in the case of investigations involving the MCF7 cell line [17–20].

Oestrogens, in common with other steroid hormones, regulate gene expression through an initial interaction with a specific cytoplasmic receptor, which then undergoes translocation to the nucleus where association with chromatin results in altered genome activity [21–23]. In recent years several compounds have been developed which act as oestrogen antagonists in the mammary gland [24–26] by interaction with the oestrogen receptor system and are therefore potentially useful in the treatment of breast cancer.

With the aim of studying the role of oestrogens and antioestrogens in controlling the growth of mammary neoplasms we have recently established 4 tumour lines, MS306, V5500, F5010 and PD6014 [27], which are serially transplantable in nude mice and have maintained the principal characteristics of the primary canine mammary tumours from which they were derived.

The present report deals with the preliminary characterisation of the oestrogen receptor from these tumour lines.

MATERIALS AND METHODS

Materials

[2,4,6,7-^{3}H]-Oestradiol (100 Ci/mmol) was obtained from Amersham International (Amersham, Bucks, U.K.). Radioinert steroids, diethylstilboestrol (DES), phenylmethylsulphonylfluoride (PMSF) and dithiothreitol (DTT) were from Sigma Chemical Company (London, U.K.). Tamoxifen and hydroxytamoxifen were gifts from I.C.I. Pharmaceuticals Ltd. (Alderley Park, Cheshire, U.K.). LY117018 was donated by Eli Lilly Ltd. (Indianapolis, U.S.A.) Scintillant 299 was supplied by the Packard Instrument Co. (Illinois, U.S.A). All other reagents were of 'Analar' grade, provided by BDH Chemicals (Poole, Dorset, U.K.).

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Trivial and systematic names of steroids and antioestrogens: oestradiol, oestra-1,3,5(10)-triene-3,17β-diol; oestriol, oestra-1,3,5(10)-triene-3,16α,17β-triol; diethylstilboestrol-3,4-bis(4-hydroxyphenyl)-3-hexene; progesterone, pregn-4-ene-3,20-dione; testosterone, 17β-hydroxy-androst-4-en-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; androsterone, 3α-hydroxy-5α-androstan-17-one; epitestosterone, 17α-hydroxy-androst-4-en-3-one; epiandrosterone, 3β-hydroxy-5α-androstan-17-one; triamcinolone acetonide, 9α-fluoro-11β,16α,17,21-tetrahydroxy-pregna-1,4-diene-3,20-dione; tamoxifen, trans-1-(p-β-dimethylaminoethoxy-phenyl)1, 2-diphenylbut-1-ene; hydroxytamoxifen, (1-(4-β-dimethylaminoethoxyphenyl)-1, hydroxyphenyl, 2-phenylbut-1-ene.

Implantation and maintenance of tumour lines

The establishment and maintenance of the MS306, V5500, F5010 and PD6014 tumour lines have been previously described [27].

Assay of oestrogen receptor

All procedures were performed at 0–4°C. Tissue was minced and then homogenised (1 g tissue:3 ml buffer) in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5 mM DTT and 1 mM PMSF containing 10% (v/v) glycerol using a Teflon–glass homogeniser. The homogenate was filtered through muslin and centrifuged at 114,000 *g* for 1 hr at 4°C in a Beckman L5-65 ultracentrifuge using an SW 50.1 6 × 5 ml swinging-bucket rotor (r_{av} 8.35 cm).

Saturation and quantitative analyses were performed under conditions ensuring maximum exchange of exogenous radioactive ligand for endogenously bound steroid. Aliquots (100 μl) of cytosol were incubated (18 hr at 4°C) with an equal volume of Tris buffer containing varying concentrations of [^{3}H]-oestradiol (ranging from 0.013 to 2.5 nM) with and without a 100-fold excess of unlabelled DES. After incubation free steroid was removed by adsorption (10 min at 4°C) using an equal volume of dextran-coated charcoal solution (0.5% Norit A, 0.05% dextran and 0.1% gelatin). Charcoal was removed by centrifugation at 800 *g* for 10 min. The radioactivity content of aliquots (200 μl) of the supernatants was determined by liquid scintillation spectrometry. Protein concentrations in supernatants were determined by the method of Lowry *et al.* [28].

Sedimentation analysis

Aliquots (1 ml) of cytosol were incubated with 2.5 nM [^{3}H]-oestradiol in the presence or absence of 250 nM unlabelled DES at 0°C for 18 hr. Free steroid was removed using dextran-coated charcoal (see above) and aliquots (400 μl) were layered onto linear sucrose density gradients [5 ml; 5–20% (w/v) sucrose] in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5 mM DDT and 1 mM PMSF. An equal volume containing bovine serum albumin (1 mg/ml) and IgG (1 mg/ml) was layered onto a parallel gradient to provide standard markers with sedimentation coefficients of 4.6 and 7.0 S respectively.

Gradients were centrifuged at 200,000 *g* for 16 hr at 4°C using an SW 50.1 6 × 5 ml swinging-bucket rotor (r_{av} 8.35 cm). Fractions (180 μl) were obtained by upward displacement with 50% (w/v) sucrose into scintillation vials and radioactivity was determined in 10 ml of scintillant 299. For location of sedimentation markers fractions were prepared by dilution with 1 ml of water and assessed spectrophotometrically at 280 nm.

Competitive binding assays

Aliquots of cytosol (90 μl) were added to tubes containing 10 μl of 50 nM [^{3}H]-oestradiol in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 10% (v/v) glycerol and 100 μl of buffer containing various concentrations of the competitors to give final concentrations of 2.5 nM for [^{3}H]-oestradiol and 0.01–1.00 μM for unlabelled competitors. Following incubation at 4°C for 18 hr, dextran-coated charcoal solution (200 μl) was added to each tube, vortexed, incubated for 10 min and centrifuged at 800 *g* for 10 min. Aliquots of the supernatant were removed for the determination of radioactivity.

RESULTS

Representative results indicating saturable binding of [^{3}H]-oestradiol in tumour cytosol are illustrated in Fig. 1(a). Competition for binding to the receptor with unlabelled DES or oestradiol (results not shown) did not show the presence of more than one steroid binding moiety. Scatchard [26] analysis (Fig. 1b) of data provided an equilibrium dissociation constant (K_D) of 33 pM and a receptor concentration of 4.5 fmol/mg cytosol protein for the example illustrated. Analysis of binding data from all tumours produced K_D values (Table 1) in the range 33–210 pM and receptor concentrations of 4.4–21.4 fmol/mg cytosol protein.

Sedimentation analysis through gradients of sucrose (Fig. 2) indicated binding of [^{3}H]-oestradiol to two components, with sedimentation coefficients of 4 S and 8 S, both of which showed degrees of displacement of [^{3}H]-oestradiol by a 100-fold excess of unlabelled DES. Competition studies emphasised the specificity of the binding proteins for natural and synthetic oestrogens and compounds with proven anti-oestrogenic properties. As shown in Fig. 3, testosterone, progesterone, triamcinolone acetonide, dihydrotestosterone, epiandrosterone, epitestosterone and androsterone did not compete for oestradiol binding sites. The most successful competitors were oestrogens and antioestrogens (Fig. 4), the hydroxylated antioestrogens being the most potent within the latter group (Fig. 4b).

DISCUSSION

The importance of analysing the functional ability of the oestrogen receptor system for the assessment of hormone sensitivity of human breast cancer has often been emphasised [30, 31]. We have previously described the establishment of 4 transplantable canine mammary tumour lines in nude mice [27]. These tumours maintain their original cellular morphology and oestradiol receptor status in all generations so far studied

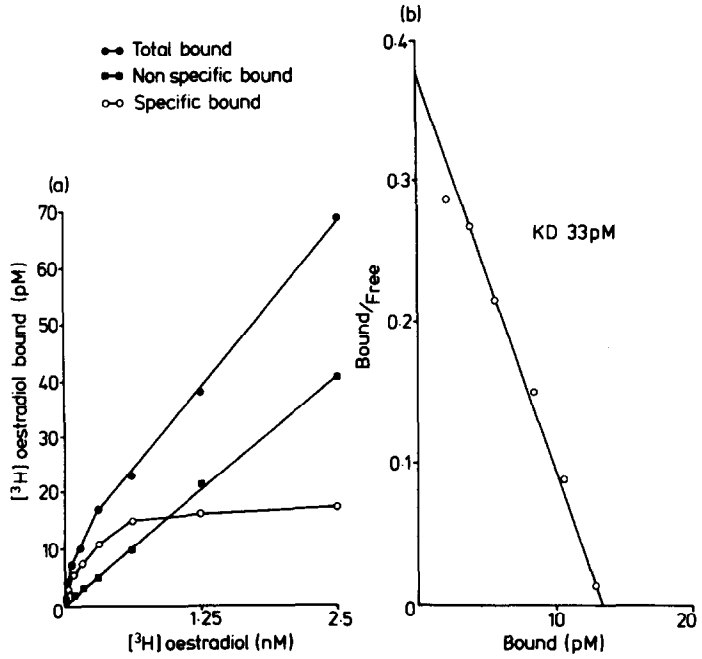

Fig. 1. Charcoal adsorption exchange assay for the measurement of cytoplasmic oestrogen receptor in the PD6014 tumour line. (a) Saturation analysis. Cytosol was incubated for 18 hr *at* 4°C *with* [^{3}H]*-oestradiol at the concentrations shown in the presence* (■) *and in the absence* (●) *of a* 100*-fold excess of non-radiolabelled DES. Specific binding of* [^{3}H]*-oestradiol was calculated by subtraction of binding values obtained in the presence and absence of DES. (b) Scatchard* [29] *analyses of data in (a).*

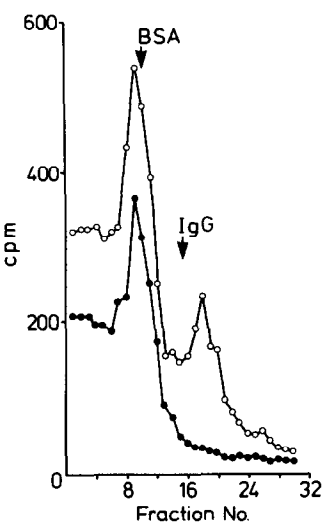

Fig. 2. Sucrose density gradient profile of [^{3}H]*-oestradiol binding in cytosol from the F5010 tumour line. Cytosol was incubated with* 2.5 nM [^{3}H]*-oestradiol in the presence* (●) *and in the absence* (○) *of* 250 nM *DES and centrifuged on* 5–20% (w/v) *linear sucrose gradients at* 200,000 *g for* 16 hr *at* 4°C. *Marker proteins were run in parallel gradients.*

and provide a ready supply of tissue for the study of oestrogen–receptor interactions in malignant mammary tissue. In this study we have undertaken the initial characterisation of the oestrogen receptor in the MS306, V5500, F5010 and PD6014 tumour lines maintained in nude mice. Scatchard analysis of the data obtained from all lines showed the receptor to have an equilibrium dissociation constant in the range of 33–210 pM, in general agreement with other workers [10, 32, 33] in studies of oestrogen receptors in mammary tumours. Sucrose density gradient analyses of cytosol from tumour lines maintained in both male and female nude mice showed characteristic 8-S and 4-S peaks for oestradiol binding, which were displaceable by excess unlabelled DES. This finding contrasts with that of Hirohashi [32], who found oestradiol receptors in the BR10 tumour cell line only when it was maintained in female hosts.

Competition for the oestradiol receptor by testosterone, dihyrotestosterone, triamcinolone acetonide, progesterone, epitestosterone, epiandrosterone and androsterone was ineffective, in agreement with the results of Watson [5] in the MXT mouse mammary tumour and the findings of Smith [34] in chick oviduct. Successful displacement of radiolabelled oestradiol was only accomplished by competition with oestrogens and the anti-oestrogens tamoxifen, hydroxytamoxifen and LY117018. The hydroxylated anti-oestrogens showed a slight superiority in competition, similar findings being reported by Nicholson and associates [32] in rat uterus and DMBA-induced mammary tumours.

Such results indicate that the oestrogen receptor described in these tumour lines is essentially the

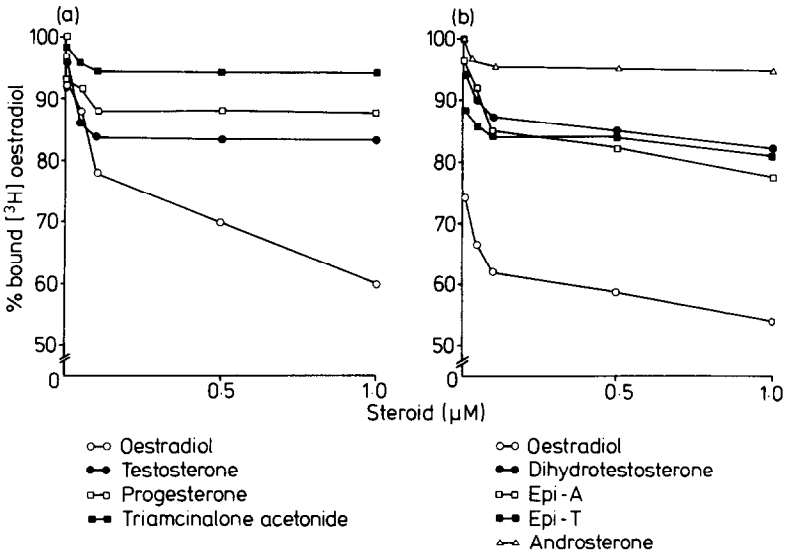

Fig. 3. Competition of [³H]-oestradiol binding in cytosol from mammary tumour lines. (a) Cytosol from the MS306 tumour line was incubated for 16 hr at 4°C with 2.5 nM [³H]-oestradiol in the presence and in the absence of increasing concentrations of non-radiolabelled oestradiol (○), testosterone (●), progesterone (□) and triamcinolone acetoninide (■). (b) Cytosol from the V5500 tumour line was similarly incubated with oestradiol (○), dihydrotestosterone (●), epiandrosterone (Epi-A) (□), epitestosterone (Epi-T) (■) and androsterone (△).

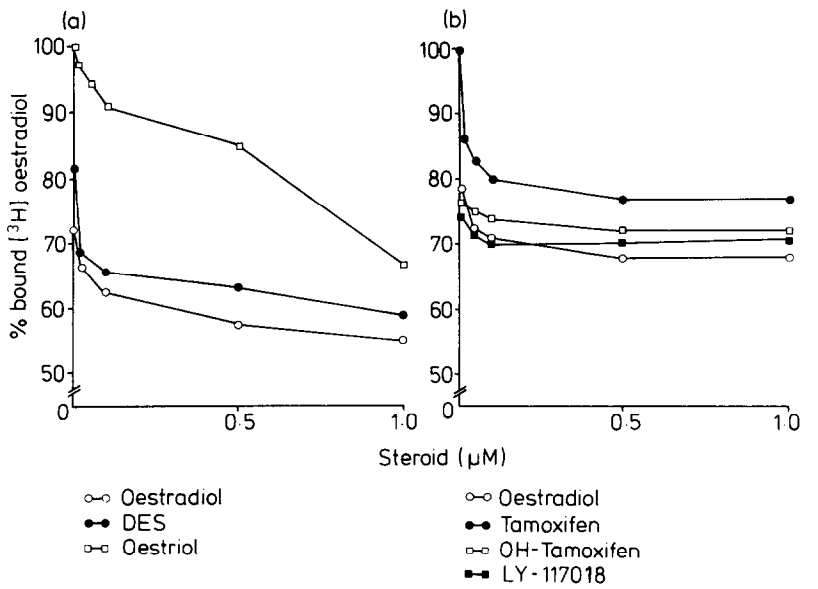

Fig. 4. Competition of [³H]-oestradiol binding in cytosol from mammary tumour lines. (a) Cytosol from the PD6014 tumour line was incubated as described in Fig. 3(a) with oestradiol (○), diethylstilboestrol (DES) (●) and oestriol (□). (b) Cytosol from the V5500 tumour line was similarly incubated with oestradiol (○), tamoxifen (●), hydroxytamoxifen (□) and LY117018 (■).

Table 1. Oestradiol receptor concentrations and equilibrium dissociation constants in mammary tumour lines during serial passages

	MS306		V5500		F5010		PD6014	
Passage	R*	K_D	R	K_D	R	K_D	R	K_D
Implant	18.3	59pM	8.4	110pM	7.9	210pM	4.4	33pM
1	13.1	147pM	13.0	ND	19.0	ND	21.4	ND
2	8.0	ND†	21.0	ND	4.7	ND	7.0	ND

*Oestradiol receptor concentration (fmol/mg cytosol protein).

†Not determined; receptor concentrations were derived from sucrose gradient or competition analyses.

same as that described in other tissues and this, in combination with the morphological stability of all four tumour lines, indicates considerable potential for this system in the continuing study of the effects of oestrogens and anti-oestrogens on tumour growth.

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