HISTOCHEMICAL DETECTION OF STEROID HORMONE RECEPTORS

and

steroid action in human tumour cell lines

HISTOCHEMISCHE BEPALING VAN STEROID HORMOON RECEPTOREN

en

werking van steroiden in humane tumor cellijnen

PROEFSCHRIFT

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Ter nagedachtenis
aan mijn moeder.

Voor mijn vader.

CONTENTS

APPENDIX PAPERS

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CHAPTER 1

GENERAL INTRODUCTION

Some cancers are hormone dependent and since the early report by Beatson (1896) on the positive effects of ovariectomy in a premenopausal patient with advanced breast cancer, the hormonal dependence of tumours has attracted much interest. The general acceptance of endocrine ablative surgery for the treatment of hormone-dependent cancers, has stimulated the use of orchidectomy in the treatment of prostate cancer (Huggins and Hodges, 1941) and the use of adrenalectomy (Huggins and Bergestal, 1952) and hypophysectomy (Luft and Olivecrona, 1953; Pearson et al, 1956) in the treatment of metastatic breast cancer in postmenopausal women. However, only 25 to 35% of all patients with advanced breast cancer show improvement following endocrine (ablation) treatment. In contrast, approximately 75 to 80% of all patients with advanced prostate carcinoma initially respond to orchidectomy or oestrogen therapy (Fergusson, 1972; Resnick and Grayhack, 1975; Scott et al, 1980), although the duration of this response as well as the time of survival of the patients are variable (Blackard et al, 1973; for a review see Waxman et al, 1985).

In a variety of target tissues steroid hormones trigger the induction of growth and/or the expression of specific gene(s). Using radiolabelled oestrogens Jensen and Jacobsen (1962) made the now "classical" observation that tissues which respond to oestrogen sequester the hormone from serum against a concentration gradient. From these and other similar experiments it was concluded, that hormone responsiveness depends on the presence of a cellular protein which binds the hormone and which has been termed the steroid hormone "receptor".

In general about 50 to 70% of the mammary tumours which contain steroid receptors ("receptor positive" tumours) appear to respond to endocrine therapy, whereas only 5 to 10% of oestrogen receptor negative tumours will respond (Lippman and Allegra, 1978; Allegra et al, 1980a; De Sombre and Jensen, 1980;

McGuire et al, 1982; Sundaram et al, 1984). Recent studies indicate that the oestrogen receptor status of the primary tumour is a good parameter for predicting the response of metastases to endocrine therapy.

The binding of the hormone to the receptor is only the initial step leading to the physiological effect of hormones. Horwitz et al (1975) and Horwitz and McGuire (1975; 1978a) have demonstrated that the induction of progesterone receptor activity in oestrogen target tissues often accompanies other oestrogen induced functions. Patients with tumours that contain both cytosolic oestrogen and progesterone receptors show the highest frequencies of positive responses (75-80%) to endocrine therapy. A summary of the studies of Lippman and Allegra, 1978; Allegra et al, 1980b; McGuire et al,1982; Cowan and Lippman, 1982; Sundaram et al, 1984; McGuire and Clark, 1985 is given in table 1.1.

Table 1.1. Levels of oestradiol (ER) and progesterone (PgR) receptors and response to endocrine therapy in breast cancer patients (means±SD).

	% of total patients in group	% of group responding to treatment
Unselected	100	30 ± 5
ER+ total	60	
ER+/PgR+	35	77 ± 4
ER+/PgR-	25	32 ± 5
ER- total	40	
ER-/PgR+	5	34 ± 18
ER-/PgR-	35	11 ± 4

In line with oestrogen receptor measurements in breast cancer, much attention has been paid to a possible correlation between the androgen receptor content in prostate cancer and the response to endocrine therapy. The clinical applicability of androgen receptor analysis in prostate cancer lays far behind that of oestrogen receptor analysis in breast cancer. Several groups have studied androgen receptor levels in cytosols (Wagner and Schulze, 1978; Ekman et al, 1979; Bradlow and Gas-

parini, 1979) and nuclear (Ghanadian et al, 1981; Trachtenberg and Walsh, 1982; Concolino et al, 1982; Gonor et al, 1984; van Aubel et al, 1985) fractions of human prostate tissues. These studies indicate that nuclear extractable and non-extractable androgen receptor concentrations may be useful indices for the prediction of the hormone dependency of prostate carcinoma.

SCOPE OF THIS THESIS

Receptor levels as such will probably never give a 100% accurate prediction about the sensitivity of tumours to hormones, due to the limitations of obtaining truely representative samples of tumour tissues as well as the inherent limitations of the receptor being only one of the parameters involved in steroid hormone action.

Both mammary and prostate carcinoma are histopathologically heterogeneous tissues and because most biochemical receptor assays are performed on tissue homogenates, it is difficult to predict responses on the basis of a single biopsy. To allow for a more precise, cell by cell analysis of the receptor content in a heterogeneous tumour specimen, histochemical methods have been developed. With such histochemical methods it might be possible to determine which or how many cells actually contain receptors and these methods might offer also a possibility for detection of receptors in small amounts of tissue or needle aspirations. One of the aims of the studies presented in this thesis was to investigate whether histochemical methods (cytofluorescence, autoradiography and immunocytochemistry) can be used for a reliable determination of steroid hormone receptors (Chapters 4, 5 and 6).

It is well known that the binding of the hormone to the receptor is only the first step in a complex pathway leading to the physiological effects of hormones. Hence to obtain a proper parameter for hormone actions it would be more meaningful to develop assays that directly measure the responsiveness of tumours with respect to growth rather than simply the presence of

receptors. In this regard the second aim of our studies was to investigate the effects of steroids on the proliferation of tumour cells in vitro, since steroids may influence cell proliferation and specific protein secretion (Chapter 7).

CHAPTER 2

ACTION OF STEROID HORMONES ON TARGET CELLS

2.1 Introduction

Steroid hormones act in a variety of target tissues to induce growth and/or the expression of specific genes. Studies concerning the mechanism of action of steroid hormones have shown that target cells for steroid hormones contain specific hormone binding proteins: the so called "steroid hormone receptors". A functional receptor is characterized by: 1) a high affinity and 2) low capacity for binding of steroid hormones, 3) steroid specificity, 4) tissue specificity and 5) it mediates an effect on target cells.

Until recently, the effect of steroid hormones on a target cell was believed to occur as follows. The steroid enters a target cell ("uptake"), followed by binding of the steroid hormone to a cytoplasmic receptor to form a steroid hormone complex. After "activation" the steroid hormone receptor complex migrates to the nucleus ("translocation") and enters the nucleus where the steroid hormone receptor complex associates with chromatin or DNA, which causes the hormonal effects. Several extensive reviews which describe these processes in greater detail have been published, e.g. King and Mainwaring, 1974; O'Malley and Birnbaumer, 1978; Van Beurden, 1977; de Boer, 1977; Baxter and Funder, 1979; Foekens, 1982; Mulder and Brinkmann, 1985; Walters, 1985; Ringold et al, 1985).

This classical "two step" model, which was proposed independently by Gorski et al (1968) and Jensen et al (1968), was based on the observed uptake and binding of radioactive oestrogens to receptors in uterine tissues from rodents. A similar model has been developed also for androgens (Liao and Fang, 1969; Mainwaring and Irving, 1973), for progestins (Milgrom et al, 1970; O'Malley et al, 1972), for glucocorticoids (Tomkins et al, 1970) and mineralocorticoids (Edelman, 1971; Scharp and Alberti, 1971). Results of autoradiographic studies have been

interpreted as supporting this model (Stumpf et al, 1968).

The apparent cytoplasmic localization of unoccupied receptors for steroid hormones has been challenged during the past few years. The results from four different experimental approaches (autoradiography, subcellular fractionation, immunocytochemistry and cellular enucleation) provided evidence for a nuclear localization of a large proportion of the unoccupied receptors (Sheridan et al, 1979; Martin and Sheridan, 1982; King and Greene, 1984; Welshons et al, 1984). This receptor appeared to be weakly associated with nuclear components until its association with the steroid converts the receptor to an active form with the ability to bind tightly to the genome (Gorski et al, 1984).

Despite a change in the model with respect to cellular localization, the process of receptor action still requires several steps. The following sections will discuss the different aspects of the interaction between the steroid hormone and its receptor which ultimately causes biochemical responses in target cells, with special emphasis on the oestrogen receptor.

2.2 Transport and entry of steroid hormones into a target cell

Steroid hormones are released from specific glands of the endocrine system into the blood stream. These steroid hormones can be transported via the plasma compartment by several proteins including serum albumin, sexhormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and progesterone binding globulin (PBG) each with a characteristic affinity and capacity. The affinity of these proteins for steroids varies from very low (serum albumin; $K_d=10^{-6}$ M) to very high (SHBG; $K_d=10^{-9}$ M). Serum albumin is present in large amounts whereas the more specific steroid binding proteins are present in smaller amounts (less than 1%) (Westphal, 1971). As long as the steroids are associated with steroid binding proteins, they are considered to be biologically inactive (Westphal, 1978). The binding proteins can influence the amount of free hormone available for receptor binding inside the cell, hence they can

be important in the control of steroid hormone action.

The blood delivers the steroids indiscriminately to target and non-target tissues. One might expect that the permeation of these steroids through the phospholipid-rich plasma membrane is facilitated by the lipophilic nature of the steroids. The precise mode of entry of steroids into cells is not yet known and conflicting results have been described (King and Mainwaring, 1974; Mainwaring, 1977; Rao et al, 1981) although most reports support the idea that the free steroid hormones in the plasma can pass the cell membrane by passive diffusion (Muller et al, 1979).

2.3 Binding of steroid by the cytoplasmic receptor ("two step" model)

It has been accepted for a long time that following their entry into the target cell, the steroids encounter proteins in the cytoplasm which have a limited number of specific high affinity binding sites: the steroid hormone "receptors". However, in prostatic epithelial cells, once free testosterone has entered the cell most of the testosterone (90%) is rapidly metabolized to DHT which is bound to the cytoplasmic receptor (King and Mainwaring, 1974; Liao et al, 1975; Coffey et al, 1978).

The specific receptors have a high affinity for the steroid (K_d=0.1 to 1 nM), with only a limited number of receptor molecules per cell (varying between 1,000 to 100,000; Baxter, 1979). Receptors are steroid and tissue specific i.e. only target tissues contain receptors whereas they are absent in non-target tissues. When isolated in hypotonic (low salt concentration) buffers cytoplasmic receptors for oestrogen, progesterone or glucocorticoids sediment on sucrose density gradients as a 8-10 S entity (Toft and Gorski, 1966; Milgrom et al, 1970; Baxter and Tomkins, 1971). At high salt concentrations (0.4 M KCl) these cytoplasmic steroid receptors disaggregate into smaller components and for example a 4 S entity has been observed for the oestrogen receptor.

When cytosolic oestrogen receptors are incubated with oestradiol they undergo an oestrogen- and temperature-dependent conformational change, called "activation" (Gorski et al, 1968; Jensen et al, 1968), which is accompanied by a 4S to 5S transition and the receptors acquire a higher affinity for oestrogens. The precise nature of the process of receptor transformation from 4 S to 5 S in the activation process is not known (Notides et al, 1985). For the glucocorticoid, progesterone and oestrogen receptor there is some evidence (Milgrom et al, 1985; Auricchio et al, 1982; 1984) that a phosphorylation step is involved in the activation process. Different factors including proteases, kinases, phosphatases, activators and inhibitors may play a role in the activation process.

2.4 Nuclear localization of unoccupied receptors

Based on the results from the studies of King and Greene (1984) and Welshons et al (1984), it has recently been proposed that the binding of oestrogen to the receptor takes place mainly in the nucleus, rather than in the cytoplasm. Two states of the oestrogen receptor, an active (liganded) and an inactive (unliganded) state are present in the nucleus. These observations have changed only the opinions about the "whereabouts" of the receptor (Schrader, 1984). Based on these results, the "two-step" model (Jensen et al, 1968) for subcellular localization of the receptor and binding of steroid requires modification. A model which can still incorporate all the important phenomena associated with the interaction of oestrogens within a target cell, is depicted in figure 1. This "new" model is similar to the model proposed for thyroid hormone uptake (Samuels et al, 1973,1976; Oppenheimer et al,1976, for a review see: Walters, 1985).

The oestrogen entering the cell initially associates with low affinity high capacity binders in the cytoplasm, which might include type II binders (Panko et al, 1978, 1981; Clark et al, 1978), and is subsequently bound in the nucleus by high affinity receptors. The secondary oestradiol binding macromole-

cule, type II, is present in large quantities and has a Kd of 70 nM. The appearance of the aggregated (8 S) state of the receptor may be the result of artificial extraction of receptors from the nucleus with hypotonic buffers. Recent studies have shown that receptors, when in the non-transformed state, exist in a complex with a 90 kD protein, a pp60src or a heat shock protein (Sullivan et al, 1985; Schuh et al, 1985; Sanchez et al, 1985). Following the binding of oestrogens to the receptor there is a conversion of the steroid hormone receptor complex to a form that associates with high affinity to one or more nuclear components. This latter form of receptor can be extracted from the nuclei only with a higher salt (0.4-0.6 M KCl) concentration. The monomeric 4 S oestrogen receptor form is transformed into a dimeric 5 S form, but it is not clear yet whether this process is either a prerequisite for or a result of the activation step (Notides et al, 1985). Not all receptors can be extracted with high-salt buffers, and a considerable part remains in the residual non-KCl extractable fraction (e.g. the matrix fraction, Barrack and Coffey, 1980).

Stumpf et al (1983) and Perrot-Applanat et al (1985) using monoclonal antibodies to the progesterone and glucocorticoid receptor, have observed that these steroid hormone receptors are localized also in the nuclei irrespective of the binding of the steroid to the receptor.

2.5 Nuclear acceptor sites and initiation of transcription

After binding of the steroid hormone to the receptor there is an increase in affinity for nuclear components. Many components have been suggested in recent years as potential nuclear acceptor sites for the receptor. These include the nuclear matrix (Barrack and Coffey, 1980, 1983; Clark and Markaverich, 1982), acidic non-histon proteins (Spelsberg et al, 1984), DNA-protein complexes and specific DNA sequences. The interaction of the steroid hormone receptor complex with the genome, resulting in an activation of selected sets of responsive genes has been reviewed by Ringold et al (1985).

Specific DNA sequences generally associated with 5' flanking regions of genes regulated by the hormones are of special interest (Payvar et al, 1981, 1983; Mulvihill, 1982; Compton et al, 1982; Groner et al, 1984). For example, hormone occupied oestrogen receptors appear to recognize discrete DNA sequences that are generally located upstream of transcriptional start sites in hormone responsive genes (Maurer, 1985; Jost et al, 1984). In contrast, specific DNA sequences both flanking and within the mouse mammary tumour virus, MMTV bind the glucocorticoid receptor (Payvar et al, 1981; Scheidereit et al, 1983), and the progesterone receptor binds selectively to chick oviduct genes (ovalbumin, conalbumin and lysozyme) (Mulvihill et al, 1982; Compton et al, 1983) and the rabbit uteroglobulin gene (Bailly et al, 1983).

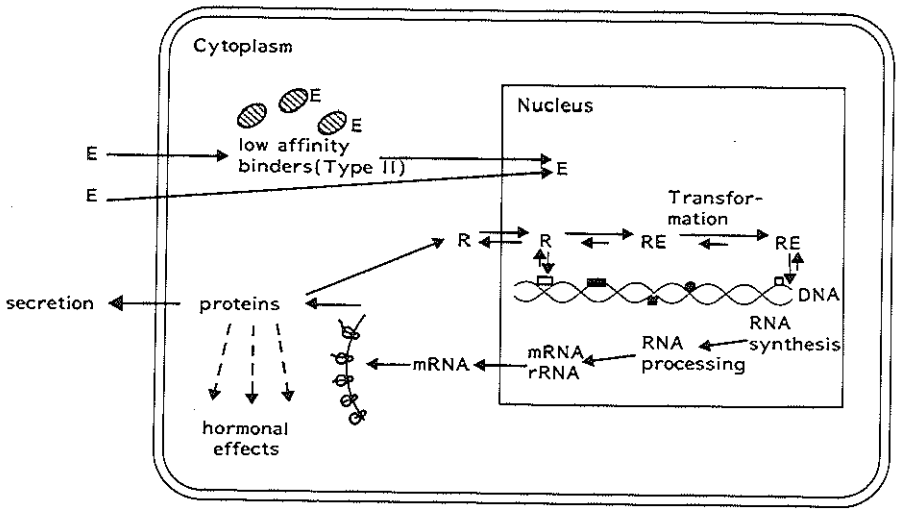


Figure 2.1. Model of oestrogen action.

The mechanism of the regulation of gene expression in eukaryotic cells via the steroid hormone receptor complex is not well understood. Two recent reports on the amino acid sequence of the human oestrogen and glucocorticoid receptor have revealed a significant regional homology among these receptors, including a cysteine rich polypeptide, which may represent the

DNA binding domain (Green et al, 1986; Greene et al, 1986). There is also a sequence homology with the v-erbA oncogene product and these receptors. This finding might assist in elucidating the molecular mechanisms underlying the steroid hormone receptor-genome interactions.

After interaction between the steroid hormone receptor complex and the genome, there are changes in the synthesis of specific mRNA's and proteins involved in the regulation of cell proliferation, differentiation and physiological functions in several tissues, as described below.

2.6 Effects of steroid hormones on mRNA and protein synthesis

The mRNA's formed as a result of the interaction of the hormone receptor complex with the genome are translated on the ribosomes and new proteins are synthesized which may cause a change in the metabolic activity of the cell. Since this thesis (Chapter 7) describes several experiments performed on the steroid hormone regulated release of proteins by cultured cells, such as the mammary tumour cell line MCF-7, and the prostate tumour cell line LNCaP (Lymph Node Carcinoma of the Prostate), some of the effects of steroids on proteins in cultured cells will be discussed. Oestrogen and androgen inducible mRNA's and proteins are of special interest, because they might serve as biochemical markers for hormone dependency of tumour cell growth and might aid in the choice of therapy.

Few reports have been published on hormone inducible mRNA's in tumour cells. The pS2 gene in MCF-7 cells represents an example of a human gene whose transcription is directly controlled by oestrogens. The level of pS2 mRNA is increased after oestradiol treatment (Brown et al, 1984).

Several studies on hormone regulated proteins have been published. Oestradiol treatment of MCF-7 cells results in higher levels of progesterone receptors in the cytosol (Horwitz et al, 1978b). In addition 4 cytosolic proteins with molecular weights of 46, 52, 54 and 60 kD can be induced by oestrogens (Mairesse et al, 1980). A 24 kD intracellular and 54 kD nuclear

protein have been observed by Adams et al (1981), and the levels of these intracellular 24 and 54 kD proteins appear to be reduced after addition of 4-hydroxytamoxifen. Interestingly, 4-hydroxytamoxifen also reduces the cell growth of the MCF-7 cells. Both plasminogen activator (Bulter et al, 1983; Ryan et al, 1984) and a 52 and a 160 kD protein were released after oestradiol treatment. The amounts of the 52 and 160 kD released proteins were also reduced by 4-hydroxytamoxifen (Westley and Rochefort, 1980). Recent studies by Vignon et al (1985, 1986) give strong evidence that this 52 kD released protein acts as a mitogen on the MCF-7 cells.

Androgens can stimulate the growth of LNCaP-cells and it has been found that prostatic acid phosphatase (PAP) was released from the LNCaP cells after androgen treatment (Horoszewicz et al, 1983; Schultz et al, 1985). Comparable to the oestrogen regulated cell growth, the androgen regulated (released) proteins might be of interest as markers for hormone regulated prostate tumour cell growth.

2.7 Steroid hormones and cell growth

Steroid hormones and their receptors appear to play an important role in the regulation of abnormal growth in various tumours and tumour cell lines (Lippman and Bolan, 1975). In the past years several in vitro systems have been developed e.g. MCF-7, ZR-75 and T47D, all derived from breast cancer metastases (Soule et al, 1973; Engel et al, 1978; Jozan et al, 1981), which might aid in the study of the molecular basis of the actions of oestrogens on breast cancer tissue.

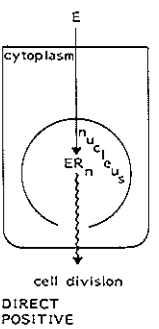
The exact mechanism of action of the steroid hormones on cell growth is not yet understood, not even for normal tissues. Stimulation of uterine growth after in vivo injection of oestrogens to immature castrated rats has been studied extensively (for review see: Walters, 1985). The picture about hormones affecting the growth of mammary (tumour) cells, however, is rather diffuse. A variety of steroids (e.g. oestrogens, androgens, progestin, glucocorticoids), iodotyrosines and polypep-

tide hormones (e.g. insulin, prolactin) and growth factors (e.g. EGF) may be involved in the regulation of breast tumour growth or metabolism (Osborne et al, 1982).

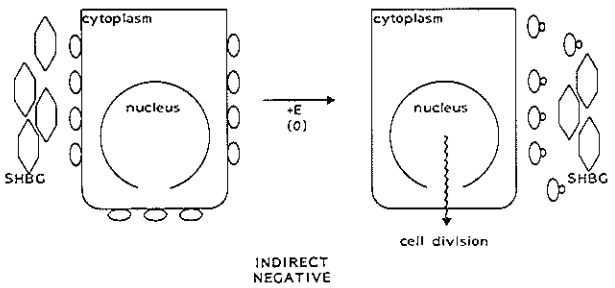
The growth stimulatory effect of oestradiol has been studied extensively in human breast cancer cell lines (e.g. Allegra and Lippman, 1980; Leung et al, 1982; Edwards et al, 1981; Chalbos et al, 1982). A direct in vitro stimulation of cell growth/proliferation by oestradiol on MCF-7 and on ZR75-1 cells has been reported by several authors (Lippman et al, 1976; Allegra and Lippman, 1980; Roos et al, 1982; Küng et al, 1983), whereas others have failed to reproduce such an effect (Horwitz et al, 1978c; Shafie, 1980; Sonnenschein and Soto, 1980a), which might be the result of differences in culture conditions. Interestingly, when cells were inoculated into animals, growth of various tumour cell lines appeared to be oestrogen responsive and sometimes the growth in vivo was dependent on oestrogens whereas in vitro growth appeared to be unresponsive to oestrogens (Shafie and Liotta, 1980; White et al, 1982).

These observed discrepancies between in vivo and in vitro growth systems have led to several hypotheses on the mechanism of oestrogen stimulated cell growth. A number of distinct working hypotheses can be considered:

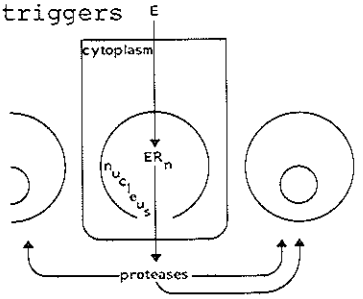
a) The "direct positive" hypothesis, which assumes that oestradiol as such triggers the multiplication of its target cells (Lippman et al, 1976; Allegra and Lippman, 1980; Dabre et al, 1983; Amara and Dannies, 1983; Stack and Gorski, 1983). A model is depicted on the right.



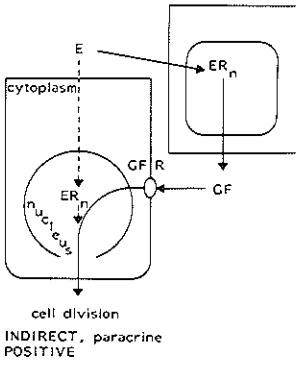
b) The "indirect negative" hypothesis (Sonnenschein and Soto 1980b) which suggests that 1) oestradiol blocks the synthesis and/or release of a specific inhibitor of oestrogen sensitive cells secreted by an intermediary organ (Laugier et al, 1983) and/or 2) plasma oestrogens neutralize the action of a putative inhibitor called estrocolyone (colyone = to inhibit) (Soto and Sonnenschein, 1983; 1984; 1985). A model is given in the figure below.



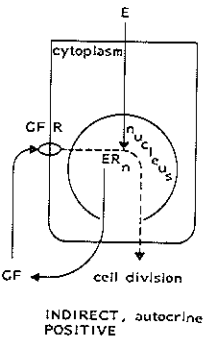
c) The "indirect positive" hypothesis (models given on the right), which suggests that oestradiol triggers 1) a facilitating factor which is released from the tumour cells, in vivo, that would ease the invasion of the surrounding space by these cells like plasminogen activator (Butler et al, 1979,1983), collagenase (Shafie and Liotta, 1980) and α-antichymotrypsine (Massot et al, 1985).



or 2) the synthesis of a growth factor which is released from an oestrogen target organ like uterus, kidney or pituitary, which exerts an endocrine regulation of mammary cell growth (Ikeda et al, 1982; Sirbasku et al, 1978).



or 3) an autocrine regulator of cell growth released from the cells (Vignon et al, 1984, 1985, 1986), which in turn causes proliferation of the oestrogen sensitive cells. There is strong evidence that the oestrogen regulated 52 kD protein (see above, 2.7) acts as a mitogen on human mammary tumour cells. (Vignon et al, 1985, 1986; Rochefort et al, 1985).



Few data have been published on human prostatic growth factors, molecular weights of 12 kD and 33-67 kD, isolated from tissue homogenates from human benign prostatic hyperplasia (Story et al, 1984; Nishi et al, 1985).

It is of interest that steroids can increase also the amount or affinity of growth factor receptors. For example progesterone increases the number of insulin receptors and the number of growth hormone and EGF receptors in T47D cells (Horwitz and Freidenburg, 1985; Murphy et al, 1985) as well as the number of EGF receptors in MCF-7 cells, whereas glucocorticoids increase the number of EGF receptors in HeLaS3 cells (Fanger et al, 1984). These EGF receptors are present on several mammary tumour cell lines (Imai et al, 1982; Fritzpatrick et al, 1984a) and Osborne (1982) have reported that EGF stimulates the growth of the human breast cancer cell line: MCF-7. Recently the induction by oestradiol of EGF- and PDGF-related polypeptides in MCF-7 cells has been described (Rozengurt et al, 1985; Dickson et al, 1986).

In vivo, oestradiol increases the number of EGF receptors (K_d=0.2 nM) in rat uteri (Mukku and Stancel, 1985), whereas EGF levels as such are also increased by oestrogens (Osborne, 1982). This might have implications for oestrogen regulated mammary tumour cell growth, possibly also for prostate cell growth in vivo and consequently for future hormonal therapies.

However, an inverse relationship has been observed between the presence of EGF receptors (with a high K_d=2 nM) and oestro-

gen receptors in human mammary tumour tissues (Fritzpatrick et al, 1984b; Sainsbury et al, 1985a,b). Although more studies will be needed, this might indicate that tumour cells in vivo react differently with respect to oestradiol inducible EGF receptors.

A suggested model which includes published data for steroid hormone regulated proteins, growth factors and their receptors is given in figure 2.2.

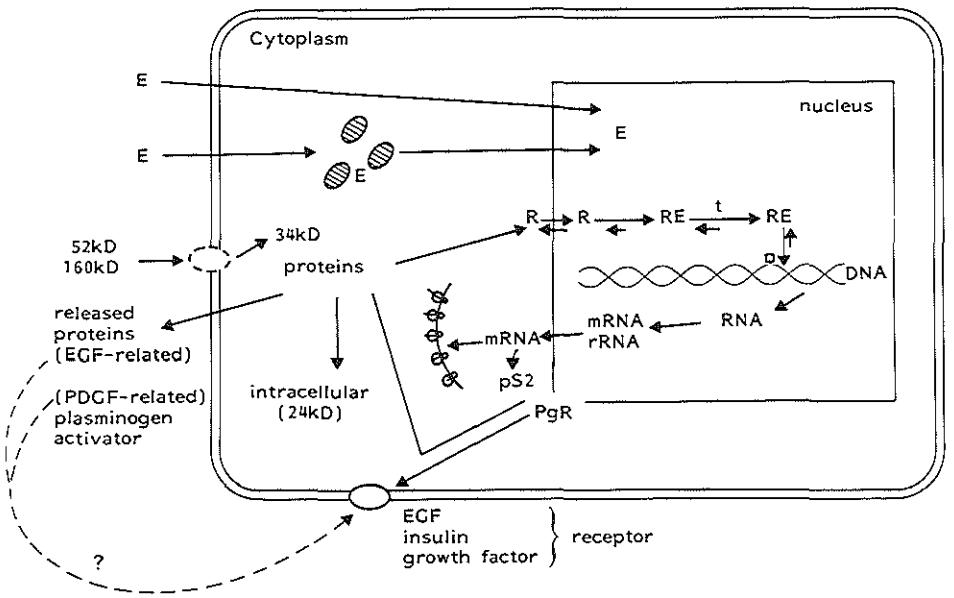


Figure 2.2. Model for the actions of steroid hormones in cultured mammary tumour cells, based on literature data.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

Several assays have been developed for the detection of steroid hormone receptors. Most of these biochemical and histochemical assays depend on either physicochemical or histo(cyto)-chemical detection of steroid hormone receptors with different (inherent) limitations, as shown in table 3.1.

Table 3.1. Biochemical and histochemical receptor assays.

Biochemical	
advantage:	quantitative, correlation with response to endocrine therapy has been studied (Chapter 1)
disadvantage:	does not discriminate between receptor positive and negative cells.
Histochemical	
advantage:	allows the discrimination between receptor positive and negative cells in a heterogeneous tumour tissue
disadvantage:	qualitative method the correlation between histochemical methods and endocrine therapy has not yet been evaluated extensively.

We have compared the biochemical and histochemical methods to investigate whether these histochemical methods are valid for receptor estimation.

3.2 Biochemical assays

The most commonly used assays for the measurement of oestrogen receptors involve the following techniques:

1) dextran coated charcoal assay (Korenman and Dukes, 1970) and Scatchard plot analysis (Scatchard, 1949),

2) sucrose density gradient centrifugation (Jensen et al, 1971),

3) (gel) electrophoresis e.g. isoelectricfocussing (Gustaffson et al, 1978) or with steroids which are covalently attached to

the receptor (Miller et al, 1983),
4) hydroxylapatite assay (Erdos et al, 1970),
5) gel filtration assays (Godefroi and Brooks, 1973).

A review of these methods has been published by Chamness and McGuire (1979). All these methods depend on the binding activity of receptor proteins for labelled steroid. The production of monoclonal antibodies against the oestrogen receptor protein permitted the development of assays which are based on direct (antigenic) recognition of the receptor molecule (Greene et al, 1980a).

3.3 Histochemical assays

Histochemical assays for the visualization of steroid hormone receptors in mammary tumours and in prostate cancer could be of great value, especially when only limited amounts of tissue are available (e.g. aspirated cells or tumour biopsies). Autoradiography, histofluorescence and immunocytochemistry have been used for the histo- or cytochemical detection of steroid hormone receptors. These methods depend either on the "direct" or "indirect" localization of the receptor, as will be discussed below.

3.3.1 Autoradiography

Autoradiography depends on the "indirect" visualization of steroid hormone receptors in tissue slices or cells via labelling with radioactive ligands, as depicted in figure 3.1. Small amounts of high affinity and specific ligands, tagged with tritium or radioactive iodine and thaw- or dry-mounting techniques have been used for the labelling of the receptor. Especially the dry-mount technique is thought to exclude sources of diffusion artifacts caused by methods like liquid fixation, embedding, wet tissue and wet emulsion mounting, thereby minimizing steroid diffusion.

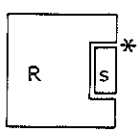


Figure 3.1. Receptor (R) with a radioactive (*) labelled steroid hormone(s).

3.3.2 Histo- and cytofluorescence

Histo- and cytofluorescence methods involve the monitoring of the cellular distribution of fluorescent labelled steroids and their derivatives. Steroids, mainly oestradiol and to a lower extent progesterone, testosterone and dihydrotestosterone, have been coupled directly through either a thiosemicarbazone, a hemisuccinate, a succinylaminohexyl or a carboxymethyloxime bridge to fluorescein isothiocyanate (FITC) or fluoresceinamine (FA). To enhance the fluorescent signal steroids were also coupled to bovine serum albumin as a carrier for more FITC or FA molecules. An example is shown in figure 3.2. Some authors used peroxidase linked oestradiol instead of fluorescent derivatives.

The naturally fluorescent plant oestrogen, coumestrol, has also been used. This coumestrol binding is difficult to monitor because considerable autofluorescence of the tissue is observed at its excitation wavelength.

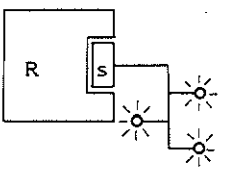


Figure 3.2. Receptor (R) with a steroid hormone (s) coupled to a fluorescent ligand.

12-oxoestradiol, also inherently fluorescent, as well as tamoxifen and 4-hydroxytamoxifen which become maximally fluorescent after UV-radiation were also studied. With a fluorescent microscope equipped with a microchannel image intensifier and a video camera detector, which enhances the signal 10,000 times and a special computer program (Martin, 1984; personal communication), to reduce the autofluorescence, the steroid receptors could be visualized.

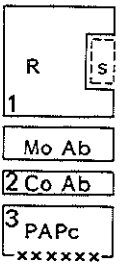
3.3.3 Immunocytochemical and immunohistochemical techniques

Initially, immunocytochemical and immunohistochemical techniques for the estimation of the intracellular localization of the receptor have used polyclonal antibodies raised against the steroid molecule (indirect immunofluorescence, Nenci et al,

1976; Pertschuk et al, 1976). The use of anti-steroid antibodies has several theoretical and technical limitations: 1) it has been suggested that once the steroid is bound to the receptor the antibody to the steroid will not bind to the receptor-bound steroid and 2) "free" steroid might be detected also with this technique.

The availability of more or less purified receptor allowed the production of poly- and monoclonal antibodies against the receptor (oestrogen, progesterone and glucocorticoid). These poly- or monoclonal antibodies raised against the receptor molecule allowed for the direct immunofluorescence technique, as shown in figure 3.3.

Figure 3.3. Receptor (R) with or without steroid (s). The monoclonal antibody raised against the human oestrogen receptor (1). Bridging antibody (2) couples the primary antibody to the peroxidase (✖) linked antibody (3). The anti-peroxidase couples to the complex. After reacting with diaminobenzidine a brown reaction product precipitates.



3.4 Biochemically characterized models

To evaluate the histochemical methods we have used cells with biochemically characterized receptor content.

Rat Leydig cells were initially used for the study of the binding of the fluorescent ligands. It appeared, however, that the oestrogen receptor in primary cultures of Leydig cells was not stable (Berns et al, 1985a; figure 3.4) and therefore Leydig cells were not used for further studies.

To circumvent the problems with primary cultured cells we have used cell lines for the histochemical studies described in this thesis. A summary of the cell lines used for the histochemical studies and the presence or absence of oestrogen and androgen receptors is given in table 3.2.

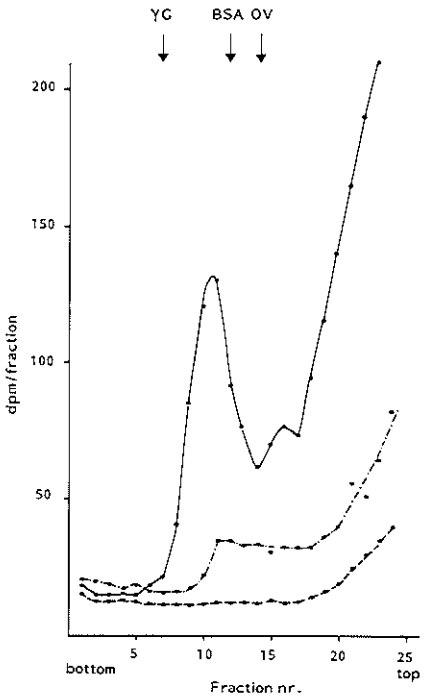


Figure 3.4. Sucrose gradient sedimentation profiles of oestrogen receptors extracted with 0.4 M KCl from nuclei of rat testicular interstitial cells after one h of cell culture (●-●) and after 24 h of culture (●- · -●). The cells were incubated for one h with 10 nM ^{3}H-E_2 in the absence or the presence of a 100-fold molar excess of DES. The sucrose gradients contained 0.4 M KCl.

Table 3.2. Receptors in cell lines.

Cell line	oestrogen receptors	androgen receptors
MCF-7	+	+
PC-93	-	+
NHIK-3025	-	+
EB-33	-	-

3.5 Steroids

(Trivial) names, specific activities and manufacturers of the steroids are given in the appendix papers. The radiochemical purity of the labelled steroids was verified by thin layer chromatography (TLC).

3.6 Fluorescent ligands

3.6.1 Oestrogenic ligands

17β-oestradiol-17-hemisuccinate-fluoresceinamine was prepared from 17β-oestradiol-hemisuccinate (E_2-HS) and fluoresceinamine. The hemisuccinate was synthesized according to Yellin, 1972 (see appendix paper 1). 17β-oestradiol-6-carboxymethyl-oxime-bovine serum albumin-fluorescein-isothiocyanate (E_2-6CMO-BSA-FITC) was obtained from Zeus Scientific (Raritan, New Jersey, U.S.A.). Properties of this compound are given in appendix paper 1. Coumestrol (1-(2,4,-dihydroxyphenyl)-6-hydroxy-3-benzofuran carboxylic acid lactone), was a gift of Dr.A.C. Notides.

3.6.2 Androgenic ligands

The preparation of testosterone-17β-hemisuccinate-bovine serum albumin-fluorescein-isothiocyanate (T-BSA-FITC) and testosterone-17β-hemisuccinate-fluoresceinamine (T-HS-FA) were based on the mixed anhydride method used by Gaetjens and Pertschuk (1980), as described in appendix paper 2. Dihydrotestosterone-17β-hemisuccinate (DHT-HS-FA) was synthesized according to Joyce et al. (1982). The structural formulas of the fluorescent ligands used in our studies are given in appendix papers 1 and 2.

3.6.3 Purification of albumin conjugate

For some experiments, free oestradiol, oestradiol-6-CMO, and free FITC were removed from 17β-oestradiol-6-CMO-BSA-FITC. One ml E_2-6-CMO-BSA-FITC (2×10^{-5}M) was layered on a Sephadex G-25 column (Pharmacia) and eluted with PBS-buffer. Fractions of 1 ml were collected.

3.7 Cells and cell culture

The MCF-7 human breast cancer cell line was provided by the Breast Cancer Animal and Human Tumour Cell Culture Bank, National Cancer Institute, National Institutes of Health, Bethes-

da, Maryland, U.S.A. The permanent human tumour cell line, PC-93 and the EB-33 cell line, both initiated from a human prostate adenocarcinoma and shown to be hormone-independent, were provided by the Department of Urology, Erasmus University, Rotterdam (Claas and van Steenbrugge, 1983; Schröder et al, 1978). The cell line NHIK-3025 was derived from an early stage of a carcinoma of the human uterine cervix (Oftebro et al, 1969). The LNCaP-FGC cell line (derived from a Lymphe Node Carcinoma of the Prostate) was a gift of Dr. J.S. Horoszewicz (Horoszewicz et al, 1983).

Detailed information on cell culture is described in the matching appendix papers.

3.8 Hormonal sensitivity and hormone metabolism of cultured cells

Assays on hormonal sensitivity and androgen metabolism of the LNCaP-FGC cells are described in appendix paper 5.

3.9 Labelling and analysis of proteins

Labelling of MCF-7 and LNCaP-FGC cells with (^{35}S)-methionine and analysis of released and intracellular proteins has been performed as described in appendix paper 5.

3.10 Biochemical assay of steroid hormone receptors in cells

Receptor isolation, sucrose density gradient centrifugation and protamine sulphate precipitation assays are described in appendix papers 1, 2 and 5.

3.11 Protein determination

The protein content of cytosols and nuclear extracts was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

3.12 DNA determination

The DNA content of the nuclear receptor preparations was determined by the method of Giles and Myers (1965) with calf thymus DNA as a standard.

3.13 Determination of relative binding affinities of the ligands

Methods for the determination of the relative binding affinities of the fluorescent oestrogenic and androgenic ligands are given in appendix papers 1 and 2 respectively. Determination of the relative binding affinity of (^{125}I)-oestradiol is described in appendix paper 3.

3.14 Histochemistry of receptors

Staining of intact cells and the "freeze damaged" cells with the fluorescent ligands is described in appendix papers 1 and 2. Autoradiography is described in appendix paper 3. Immunocytochemistry and the combined immunocytochemistry and autoradiography are described in appendix paper 4.

3.15 Microscopy

The cells were examined under a fluorescence microscope (Leitz Orthoplan with epifluorescence: equipped with a 100 W mercury bulb and an Orthomat-camera), as described in appendix paper 1.

CHAPTER 4

COMPARISON OF BIOCHEMICAL RECEPTOR ESTIMATIONS AND HISTOCHEMICAL STAINING WITH FLUORESCENT STEROID HORMONE DERIVATIVES

4.1 Introduction

Histochemical methods for the visual detection of steroid hormone receptors in both mammary tumours and in prostatic carcinoma could be of great value, especially when only limited amounts of tissue are available. When compared to the "conventional" biochemical methods, histochemical methods might be faster and cheaper, they would permit more precise, cell-by-cell analysis and they might be applied to small amounts of tissue, or even aspirated cells. With respect to the usefulness of fluorescent steroid conjugates for direct visualization of steroid hormone receptors in cells or in tissues, contradictory results have been published, e.g. Dandliker et al, 1978; Pertschuk et al, 1978a,b, 1979; 1980a,b; Barrows et al, 1980; Chamness et al, 1980; Daxenbichler et al, 1980, 1984; Fetissof et al, 1980; Lee, 1980, 1981; McCarthy et al,1980; Nenci et al, 1980; Chamness and McGuire, 1982; Fisher et al, 1982; Joyce et al, 1982; Lindeman and van Marle, 1982; Panko et al, 1982; Underwood et al, 1982; Martin et al, 1983; Lämmel et al, 1983; Berns et al, 1984a,b,c,d; Parl et al, 1984; Binder et al, 1984; Bergquist et al, 1985 and Janssens et al, 1985.

In this chapter our attempts to use oestrogens and androgens coupled through either BSA or a hemisuccinate bridge to the fluorescent ligands in a histochemical assay for detection of binding proteins in receptor-positive and -negative cells are described. For these studies we used cell lines with biochemically characterized oestrogen or androgen receptor content. Martin et al (1982, 1983) reported on the visualization of oestrogen receptors in target cells using inherently fluorescent ligands, like coumestrol and 12-oxoestradiol, or 4-hydroxytamoxifen and image intensification. Since 4-hydroxytamoxifen is a metabolite of tamoxifen, an anti-oestrogen widely

used in hormonal therapy of breast cancer, the possibility of visualizing oestrogen receptors using 4-hydroxytamoxifen is particularly interesting. Hence, we have attempted also to visualize the oestrogen receptors using 4-hydroxytamoxifen and image intensification.

Variability in results may be caused by ill-defined tissue preparations and incubation techniques or impure preparation of steroid conjugates. Tissue sections contain a variety of intact cells, damaged cells and dead cells, and during fixation and incubation denaturation and diffusion of proteins may occur. To prevent these difficulties, the experiments in our investigation were performed with intact cultured cells or with cells which were reproducibly damaged by a standard procedure. For a reliable localization of steroids several criteria should be fulfilled (Table 4.1).

Table 4.1. Criteria which fluorescent conjugated steroids have to fulfill for binding to receptors.

1. High relative binding affinity.
2. Pure and stable.
3. Competition with the natural ligand.
4. Low non-specific binding.
5. Saturable binding.
6. Concentration of binding sites must be in agreement with the biochemically measured concentration.

4.2 Results and discussion

4.2.1 Receptor content

The oestrogen and androgen receptor content in the nuclear extracts and cytosols of MCF-7, PC-93, NHIK-3025 and EB-33 cells have been published in appendix papers 1 and 2.

4.2.2 Relative binding affinities of the fluorescent ligands

The relative binding affinities of the fluorescent ligands

for the oestrogen receptors in the cytosol of the uterus from an ovariectomized rat were calculated as the ratio of concentration of oestradiol and the fluorescent ligands required to reduce specific binding of labelled oestradiol by 50%. The relative binding affinities of the fluorescent ligands for the androgen receptors in the cytosol of the prostate from a castrated rat were calculated as the ratio of the concentrations of methyltrienolone or the fluorescent ligands required to reduce specific binding of (^{3}H)-methyltrienolone by 50%. The fluorescent ligands show very low relative binding affinities (Table 4.2).

Table 4.2. Relative binding affinities (RBA) of the fluorescent ligands.

Oestrogenic ligands	RBA (%)	Androgenic ligands	RBA (%)
Oestradiol (E_2)	100	Methyltrienolone	100
E2-6-CMO-BSA-FITC *	4.3	T-17β-HS-BSA-FITC	0.1
E2-6-CMO-BSA-FITC **	1.8	T-17β-HS-FA	0.1
E2-17β-HS-FA	0.1	DHT-17β-HS-FA	4
Coumestrol	1.4		

* Commercial preparation, used without purification
** Free oestradiol partially removed

4.2.3 Stability and purity of the ligand E_2-6CMO-BSA-FITC

Free oestradiol, oestradiol-6-CMO and free FITC were removed from the E_2-6-CMO-BSA-FITC conjugate as described in chapter 3.6.3. Two fluorescent peaks were observed, peak 1 contained the E_2-6-CMO-BSA-FITC conjugate, whereas peak 2 contained 79% of the FITC molecules, indicating that most of the FITC molecules were not bound to the albumin conjugate. An elution pattern is shown in figure 4.1. The concentration of free oestradiol, not bound to the conjugate was 18.8 nM which is more than sufficient to saturate the oestrogen receptor. It is not known whether the product was instable and had decomposed during the transport or whether is was impure at the time of dispatch. De Potter et al (1985) observed a similar impurity

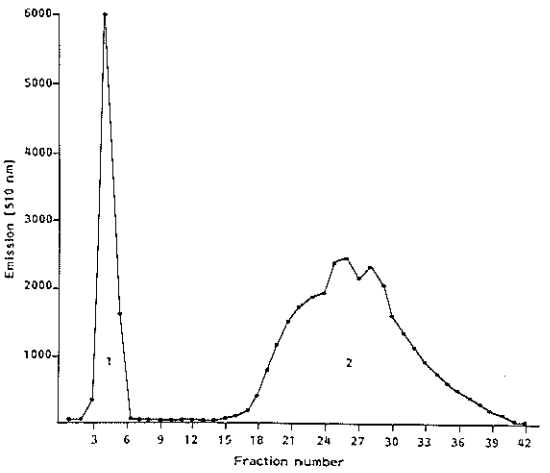


Figure 4.1. Elution pattern of E_2-6-CMO-BSA-FITC with PBS-buffer on a Sephadex G-25 gelfiltration column (fractions of 1 ml were collected).

after analysis of this product. From these results we have concluded that this FITC preparation was not pure.

4.2.4 Histochemical studies

4.2.4.1 Intact cells

Intact cells were stained with 10^{-5}, 10^{-7} and 10^{-9} M of the fluorescent ligand. No difference in fluorescent staining was observed between steroid hormone receptor- positive and -negative cells, after addition of the hemisuccinate conjugated derivatives (examples are shown in appendix papers 1 and 2) or coumestrol. Addition of the native ligand at a concentration of 10^{-7} M oestradiol to the oestrogen-receptor containing cells or testosterone or dihydrotestosterone to the androgen receptor containing cells to the staining solution, produced no decrease in intensity and again no difference in fluorescent cytoplasmic staining between receptor-positive and -negative cells was observed. No fluorescence was observed with the BSA-linked derivatives, which is probably due to the impermeability of the cell membrane for the albumin derivative. Pretreatment of

intact cells for 24 hours prior to staining in steroid hormone free medium, did not affect the fluorescence. Addition of FITC, the fluorescent moiety of the albumin steroid complex, also resulted in a fluorescent staining of the cells. This green stain differed from the E_2-6-CMO-BSA-FITC or T-17-HS-BSA-FITC stain, which was bright apple green. Addition of fluoresceinamine, the reagent used for synthesis of E_2-HS-FA, T-HS-FA or DHT-HS-FA to the intact cells, did not reveal any fluorescence (see table 4.3).

4.2.4.2 "Freeze-damaged" cells

"Freeze-damaged" cells were stained also with 10^{-5}, 10^{-7} or 10^{-9}M of the fluorescent ligands. Again no difference in fluorescent staining was observed between the receptor-positive and -negative cells. All cell types stained with the HS-linked derivatives showed a cytoplasmic fluorescence. When stained with the BSA-linked derivative, all cell lines revealed cytoplasmic and more intense nuclear fluorescence (examples are shown in appendix papers 1 and 2), whereas with coumestrol no fluorescence was observed at all. Addition of the ligand at a concentration of 10^{-7}M did not affect the staining pattern. With unconjugated FITC and FA, results with damaged cells were the same as for the intact cells (see table 4.3).

From these experiments it can be concluded, that the stain-

Table 4.3. Results of the histochemical assay.
For <u>all</u> cell lines used in this study.

Intact cells:	- Non protein conjugates:	cytoplasmic fluorescence
	- BSA-derivatives :	no fluorescence
"Freeze-damaged" cells:	- Non protein conjugates:	cytoplasmic fluorescence
	- BSA derivatives :	nuclear fluorescence, predominates

* Staining is independent of the presence of receptors.
** Staining is not suppressed with excess native ligand (oestrogen or androgen.

ing patterns observed for the fluorescent ligands, under different experimental conditions, showed no relation to the oestrogen or androgen receptors estimated by biochemical methods. These results are in agreement with recently published observations, some of which obtained with tissue sections of tumours (McCarthy et al,1980; Chamness et al, 1980; Joyce et al, 1982 and Lämmel et al, 1983). For example Joyce et al (1982) showed for oestrogen labelled fluorescent conjugates (oestrogens coupled through a variety of short spacers) that in thin sections of breast tumour tissue these compounds did not bind to the classical oestrogen receptors. Moreover, Lämmel et al (1983) reported for dihydrotestosterone labelled FITC conjugates with a variety of short spacers, that it was not possible to demonstrate androgen receptors in tissue slices obtained from human prostatic carcinomas and human benign prostatic hyperplasia.

The anti-oestrogen 4-hydroxytamoxifen, which has a high relative affinity (about 100% compared to E_2) for the oestrogen receptor, was used to detect specific binding sites in the MCF-7 cells. The MCF-7 cells were incubated with concentrations of 10^{-9} to 10^{-7} M of 4-hydroxytamoxifen for 1 hour at 37°C. After ultraviolet irradiation, which makes the ligand maximally fluorescent, the MCF-7 cells were viewed under a fluorescence microscope equipped with a microchannel image intensifier and a video camera detector. This combination enhances the sensitivity of detection about 10,000 times. Under these conditions mainly nuclear fluorescence can be expected, but we observed only cytoplasmic fluorescence. Even when the cells were grown in special media, to reduce the autofluorescence which might interfere too much for a proper visualization of the receptor, we also observed cytoplasmic fluorescence (this study was performed in collaboration with Dr. H. Tanke, University of Leiden). Martin et al (1983) observed some nuclear localization 4-hydroxytamoxifen but the image quality was poor because of a high background fluorescence. To improve the quality of the fluorescence, they were using special, more appropriate, barrier filters and digital image recording and background

subtraction with the use of special computer programs (Martin, personal communication). Since these programs were not available to us, we have not been able to perform similar experiments.

Clark et al (1978) and Panko et al (1982) claimed that 2-10 times more so-called "type II binding sites" may be present in breast carcinomas. However, with standard fluorescence microscopy one would probably not detect either the 10,000 true receptor molecules or the 100,000 "type II binding sites" in a cell, because the concentrations of these sites are below the limit of detection of fluorescein molecules in a cell (Chamness and McGuire, 1982). The fluorescence observed in the cells used in the present study must therefore be due to binding of the ligands to low affinity binding sites present in higher concentrations than the type II binding sites. We have analyzed by electrophoresis the different types of proteins responsible for binding of the fluorescent ligands at ligand concentrations of 10^{-6} to 10^{-7} M. The ligand E_2-HS-FA was used with MCF-7 cells and non-denaturing polyacrylamide gel electrophoresis was performed to characterize the proteins. We observed one intense band of fluorescence after electrophoresis. The fluorescence in this band was not reduced after incubation of the cells with E_2-HS-FA and 10^{-6}M E_2. This is a further indication for the non-specific character of the protein(s) that bind(s) the fluorescent steroids. An example is shown in figure 4.2. Parl et al (1984) observed that after isoelectric focussing of MCF-7 and T47D cell cytosol incubated in the presence of 10 nM 17-FE (N-fluoresceino-N'-17β -estradiol-hemisuccinate-ethyl-thiourea) that the binding of the fluorescein conjugate is merely to a protein species which did not bind 17β-(^{3}H)-E_2. Some minor binding occured to a protein which could be the oestrogen receptor.

From the results of this study it appears also that the concentration of these low affinity binding sites, estimated with fluorescent ligands, is not related to the concentration of true receptors and cannot be used as a basis for discrimination between oestrogen and androgen receptor positive and -negative cells.

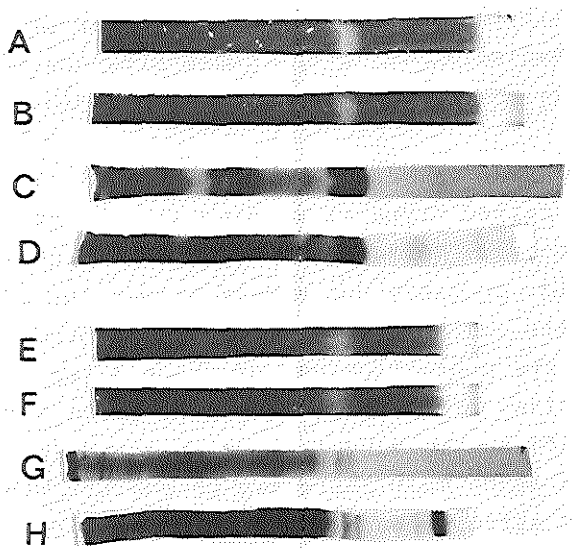


Figure 4.2. Non-denaturating PAGE (8.4% acrylamide with 10% glycerol, anode, left; cathode, right) of human plasma (A-D) or cytosol of MCF-7 cells (E-H) incubated for 1 hour with E_2-HS-FA ± 10^{-6} M E_2 at room temperature and 37°C respectively. Lanes A, B E and F represent fluorescent staining (150 μg protein per lane). Lanes C and G were stained with Coommassie (50 μg protein per lane). Lanes D and H were silverstained (50 μg protein per lane).

4.3 Conclusions

From the results presented in this chapter we conclude that:

- Both oestrogen or androgen receptor-positive and receptor negative cells are stained with the fluorescent ligands.
- Receptors, for oestrogens and androgens, cannot be visualized with the low affinity fluorescent ligands, used in the present study.
- Fluorescence of the cells is probably due to binding to low affinity binding sites.
- The presence of the observed low affinity binding sites appears not to be related to the presence or absence of the oestrogen or androgen receptors.

CHAPTER 5

AUTORADIOGRAPHY OF THE OESTROGEN RECEPTOR IN CELLS AND TISSUE SLICES, USING (^{125}I)-LABELLED OESTRADIOL

5.1 Introduction

Using autoradiography steroid receptors in cells and tissue slices can be "visualized indirectly" using small amounts of specific high affinity ligands, tagged either with radioactive hydrogen, carbon or iodine (Chapter 3). Autoradiographic methods which require the fixation and dehydration of tissue slices or cultured cells are inadequate for the study of steroid hormones, since artifacts due to either diffusion, translocation or extraction of the steroid would occur. The "dry-mounting" of the cells or sections on the photographic emulsion, prevents the diffusion of label towards the photogragraphic emulsion.

With this technique, Stumpf and Roth (1966) were the first to demonstrate that after in vivo incubation of tissue slices with (^{3}H)-oestradiol, most of the (^{3}H)-oestradiol is localized over the cell nucleus of a variety of steroid target tissues. Several groups have confirmed this observation and this was extended to include other classes of steroid hormone receptors (e.g. Roth and Stumpf, 1969; Stumpf and Sar, 1976; Weiller et al, 1976; Verhoeven et al, 1980; Sheridan et al, 1981; Schleicher et al, 1984). This information on the subcellular distribution of steroid hormone receptors could not have been obtained by biochemical procedures. The soluble steroids are retained also in situ in freeze-dried cells or cryostat sections of tissues (Stumpf and Roth, 1964). Moreover, the integrity of the cells, grown in monolayer culture, or the tissue is preserved throughout the radioautographical process. This "dry-mount" technique, however, is very laborious to perform. Hence, we have also evaluated the autoradiographic technique described by Frederik (1977), which is less laborious to perform and we have compared this method

with the method used by Stumpf et al (1968). The technique described by Frederik allows the protection of cells or tissue slices with a carbon layer, which is applied on the preparations. This carbon layer should prevent (minimize) translocation of diffusable substances during the process of autoradiography. The emulsion is layered on the cells or tissue slices by the "dip" or "loop" technique (Caro and van Tubergen, 1962).

In general, tritiated ligands have been employed to visualize steroid binding components in cell populations. Using such tritiated ligands, with a high specific activity (SA: 150 Ci/mmol) it is possible to detect steroid binding at hormone concentrations (0.01-1 nM) of physiological significance (Stumpf and Roth, 1966; Stumpf and Sar, 1976). A great disadvantage of these autoradiographic studies is that they require long exposure times (weeks to months) at low temperature, making this technique inappropriate for wide-spread clinical use.

Iodinated ligands have a higher specific activity and shorter exposure times are required for autoradiography. The synthesis of 16α-(^{125}I)-oestradiol with a specific activity of about 2000 Ci/mmol (Hochberg, 1979), 16α-(^{125}I)-5α-dihydrotestosterone (Hoyte et al, 1982) and 16α-(^{125}I)-testosterone (Symes et al, 1985) with a half life of 60 days might allow receptors to be measured with a greater sensitivity and in case of autoradiography, shorter exposure times can be used. In vivo studies with these labelled compounds revealed that the (^{125}I)-E_2 had not lost its oestrogenic activity and that (^{125}I)-E_2 concentrates in the rat uterus (Hochberg, 1979). The iodinated 5α-dihydrotestosterone and testosterone preparations, however, were not active in vivo. The testosterone derivative did not even accumulate against the blood gradient in the target tissues after in vivo labelling. The relative binding affinity of the iodinated 5α-dihydrotestosterone was 0.01% and no specific binding was detected when this 16α-iodo analog was incubated with prostate cytosol (Hoyte et al, 1982; Symes et al, 1985). According to the criteria listed by Williams (1977), (^{125}I)-oestradiol might be very suitable for

autoradiographic studies. In this respect, we have used (^{125}I)-oestradiol for autoradiography of the oestrogen receptor in cells and tissue sections.

5.2 Methods

Tissue sections or cells were labelled with (^{125}I)-oestradiol, freeze-dried, covered with a carbon layer, exposed to the photographic emulsion and developed. Thereafter, the preparations were studied with light microscopy. The results, obtained with this technique were inconsistent. In addition, we have used the "dry-mount" autoradiographic technique to study the localization of (^{125}I)-oestrogen binding proteins (appendix paper 3). With this dry-mount autoradiographic technique it is possible to localize oestrogen receptors in cultured cells and in cells from tissue sections within 24 hours.

5.3 Results and discussion

5.3.1 Biochemical studies

The introduction of the iodine atom on the 16α-position of oestradiol might influence the transport of the labelled oestradiol through the cell membrane or the binding of the ligand to the receptor molecule. Using (^{125}I)-labelled oestradiol, Hochberg (1979) and Hochberg and Rossner (1980) observed that the labelled oestradiol concentrates in the rat uterus after in vivo injection of the steroid. Further experiments revealed that this γ-emitting isotope specifically binds to the oestrogen receptor in rat uterus cytosol. The binding of (^{125}I)-oestradiol to the oestrogen receptor in the nuclei, however, was not investigated. The amount of binding sites was also not determined and could therefore not be compared with the amount of binding sites measured after incubation with the tritiated ligand. We have first compared the binding of (^{125}I)-oestradiol and (^{3}H)-oestradiol to the oestrogen receptor in

intact MCF-7 cells and to the oestrogen receptor from cytosol of rat and calf uteri. After incubation of the MCF-7 cells with either (^{125}I)-E_2 or (^{3}H)-E_2, the nuclei were isolated and extracted in a buffer containing 0.4 M KCl. The extracts were layered on sucrose gradients (also containing 0.4 M KCl). Sucrose gradient centrifugation of the nuclear extracts revealed a peak sedimenting at 4S when the cells were incubated either with (^{3}H)-E_2 or (^{125}I)-E_2. Similar qualitative characteristics were observed after analysis on sucrose gradients of calf uterus cytosol incubated either with (^{3}H)-E_2 or (^{125}I)-E_2. After incubation of calf uterus cytosol with (^{125}I)-E_2 or (^{3}H)-E_2 for 2 hours, comparable amounts of binding sites were measured. However, using rat uterus cytosol the amount of binding sites measured was about 70% of the amount found with (^{3}H)-E_2. This was a matter of concern, but Grill et al (1983) observed that the kinetics of binding to the human oestrogen receptor of (^{3}H)-E_2 and (^{125}I)-E_2 differed at 4°C. With (^{3}H)-E_2 the equilibrium is already reached after 3 hours, whereas with (^{125}I)-E_2 9 hours are needed. Studies on binding kinetics for rat and calf uterus receptors were not included in this study. Hence, it is possible that the incubation period used in our study (2 hours) might be too short resulting in a lower amount of binding sites measured after incubation with (^{125}I)-E_2. Competition studies with oestradiol revealed that the relative binding affinity of (^{125}I)-E_2 for the oestrogen receptor is about 75%, whereas the relative binding affinity of (^{3}H)-E_2 for the oestrogen receptor is set as 100% (see appendix paper 3). Grill et al (1982), Tercero et al (1981) and Duffy et al (1982) also observed that (^{125}I)-E_2 and (^{3}H)-E_2 had similar binding characteristics with respect to affinity and the amount of oestrogen receptor sites detected.

From these studies and from the results described in appendix paper 3, we conclude that the introduction of iodine on the 16α-position of oestradiol appears not to alter significantly the binding of the oestrogen to its receptor.

5.3.2 Histochemical studies

In the preceding section it was concluded that (^{125}I)-E_2 binds to the oestrogen receptor. This ligand has a very high specific activity (2000 Ci/mmol) which may reduce the exposure time needed for autoradiography from months to days. An advantage of this ligand is also that it is not converted into the estrone derivative by 17β-hydroxysteroid dehydrogenase (Grill et al, 1983). Hence, if grains are observed above the cells, these are probably due to (^{125}I)-E_2 bound to the (receptor) protein.

Initial studies using autoradiography with (^{125}I)-E_2 were performed according to Frederik (1977). Briefly, intact MCF-7 cells, cultured on slides, were incubated with (^{125}I)-E_2 for 1 hour at 37°C, washed with PBS-buffer and dipped in liquid Freon 22. Freon 22 has a high cooling velocity (about 1500°C/sec at a temperature interval of -20 to -80°C) which is necessary for a proper histological integrity. The preparations were stored in liquid nitrogen and subsequently freeze-dried for 24 hours. After freeze-drying, a thin layer of carbon (which should prevent translocation of the steroid) was applied on the preparations and they were dipped in an Ilford K2 emulsion. The preparations were stored at -4°C for 16-28 hours in a black plastic slide box. Sheets of lead were placed between slides to prevent emission of particles to the emulsion on other slides. After development of the slides we observed grains mainly above the cells and dispersed around the cells. The background was high. To reduce this background, we included two washing steps with 1% BSA in PBS in our washing procedures. This procedure reduced the background grains to some extent, but not sufficiently. Sheridan et al (1979) and Martin and Sheridan (1982) reported that a washing step with diluted antisera generated against the steroid could greatly reduce the autoradiographic background. Since it has been shown by Hochberg and Rossner (1980) that iodinated oestradiol tightly binds to an anti-oestradiol antibody raised against oestradiol-derivatives at carbons 3, 6 and 17, we have

also included two washing steps with an antibody raised against oestradiol (dilution 1:1000). We also included a chase with a 100-fold molar excess unlabelled oestradiol to reduce the background and to exchange non-specifically bound label, as described by Weiller et al (1976). This combination of washing procedures resulted in a low acceptable amount of background grains (as described in appendix paper 3). We still observed, however, a disperse pattern of grains above and around the cells. Due to the labelling procedure used, one would expect grains mainly above the nucleus of the cells.

In our routine procedure some of the preparations were counterstained with haematoxylin and azofloxin. We were surprised to see that the cells were stained, because it has been described that the carbon layer should prevent translocation of the diffusable substances (like staining solutions and steroids!). The latter results indicated, however, that the carbon layer does not prevent the diffusion of soluble substances. To investigate this, a control experiment was performed. In this experiment the cells were incubated with a relative high amount (5 nM) (^{125}I)-E_2 and processed for autoradiography as described above. After developing we observed a black dot, representing the labelled cells and a smear, representing the (^{125}I)-E_2 which was dissolved in the emulsion and transported with the liquid photographic emulsion during the "dipping" procedure (not shown). These results led to the conclusion that the carbon layer does not protect sufficiently against the diffusion of (^{125}I)-oestradiol. This "carbon technique" has also been questioned by others. Williams (1982) observed also that the carbon technique does not guarantee the proper localization of low molecular weight diffusable substances at the ultrastructural level using the technique described by Frederik and Klepper (1976).

Because the "carbon technique" appeared to be unsuitable for our purpose, we repeated the experiments as described above using the "dry-mount" autoradiographic technique as described by Stumpf (1966) instead of the "carbon technique". Dry-mounting of freeze-dried sections or cells excludes all known sour-

ces of translocation such as liquid fixation, embedding, de-embedding, rehydratation, thawing, wet section mounting and application of liquid emulsion or wet stripping film.

MCF-7 cells cultured on slides were incubated, washed and freeze-dried as described in the previous section. These freeze-dried cells were transferred from the glass slide to a slide which was previously coated with the photographic emulsion. The emulsion with adhered cells was kept at 4°C. After exposure and development, the cells were counter stained and examined under a microscope. The silver grains were localized only over the MCF-7 cells. We also observed heterogeneity of labelling (shown in appendix paper 3).

Control experiments, with a 100-fold molar excess of unlabelled oestradiol, designed to assess non-specific binding, revealed only a very limited amount of silver grains over the cells (also shown in appendix paper 3). Macrophages and PC-93 cells, which do not contain oestrogen receptors (as estimated with biochemical techniques), were also included in this study. Macrophages are consistently smaller in size when compared to MCF-7 cells and could therefore be used in a co-culture experiment with MCF-7 cells. After incubation with (^{125}I)-E_2, macrophages did not retain the label and did not reveal silver grains, in contrast to the MCF-7 cells (shown in appendix paper 3). PC-93 cells also did not retain the label and revealed no grains above the cells.

From these results we have concluded that oestrogen receptor-positive cells retain the iodinated label resulting in a varying amount of silver grains, whereas oestrogen receptor-negative cells do not.

These results are in agreement with the results of Martin and Sheridan (1980). Using tritiated oestradiol, they observed a localization of silver grains above the MCF-7 cells and other oestrogen receptor-positive cells, whereas oestrogen receptor-negative cells appeared devoid of silver grains above their cells. They also observed heterogeneity in labelling. The silver grains were localized over the nuclei.

In addition, we have incubated tissue sections of rat and

calf uterus with (^{125}I)-oestradiol without or with a 100-fold molar excess of unlabelled oestradiol. These sections were washed, freeze-dried and dry-mounted by pressing the glass slide to the emulsion coated slide between forefinger and thumb. After exposure, development and staining, we observed a predominant labelling of the endometrial gland cells and stromal cells (appendix paper 3). This labelling could be suppressed by a 100-fold molar excess of unlabelled oestradiol, indicating that this technique reveals only receptor-bound oestradiol. The results obtained with (^{125}I)-oestradiol autoradiography are compared with the results obtained with tritiated oestradiol in the next section.

Stumpf (1968) observed, after a subcutaneous injection of (^{3}H)-E_2 into a rat, a nuclear concentration of (^{3}H)-E_2 in the cells of the epithelium of the luman and the glands, the substantia propria and muscularis. He observed variations in silver grain density and a small population of cells did not show radioactivity. This observation was explained by decomposition characteristics of tritium or variations in the oestradiol binding affinity, perhaps in relation to changes in the cell cycle. Since it is not (always) possible to inject animals or humans with tritiated steroids other labelling techniques have been developed. For example, small blocks of tissue (1 mm thick) were incubated in medium equilibrated with 95% O_2 and 5% CO_2 and tritium-labelled oestradiol. Tchernitchin et al (1973) demonstrated that after incubation of tissue blocks of human endometrium, there is a specific uptake of radioactivity by glandular and luminal epithelial cells as well as stromal cells. Similar experiments using mouse uterus and human endocervix also revealed a nuclear localization of silver grains on epithelial and stromal cells (Shannon et al, 1982). This technique was applied also to human mammary tumour tissue blocks. Buell and Tremblay (1983, 1985) observed that human mammary cancer is composed of a heterogeneous population of labelled and unlabelled tumour cells. They also observed that the number of grains varied among putative target cells and that

the relative percentage of labelled cells differed from area to area. In addition to tumour cells, some specific labelling was identified in non-neoplastic ducts whereas other ducts were devoid of grains. There was an overall agreement between this autoradiographic technique and the biochemical assay of 92%, but if borderline cases are included the overall agreement drops to 75%.

The observed variability in uptake and retention of (^{3}H)-E_2 is not considered to be a technical artifact like poor penetration of the ligand into the tissue blocks during incubation, since cells with relatively few or without nuclear grains could be identified adjacent to strongly labelled cells. This observation makes it very likely that diffusion of the steroid is adequate. We also observed a heterogeneous labelling of cells in tissue sections (4-5 μm) and in cells cultured on slides (where diffusion should be easy). As a result from these studies we conclude that artifacts of in vitro autoradiographic techniques are not responsible for the heterogeneity in labelling which we observed.

5.4 Conclusions

- (^{125}I)-E_2 when compared to (^{3}H)-E_2 shows similar characteristics with respect to qualitative and quantitative binding to the oestrogen receptor (^{125}I)-E_2 has a high relative binding affinity for the oestrogen receptor.
- Dry mount autoradiography, using (^{125}I)-oestradiol provides a rapid and sensitive detection of oestrogen receptors in cells and tissue sections.
- It is possible to discriminate between oestrogen receptor-positive and -negative cells with this technique.
- The heterogeneity of cells with respect to the distribution of receptors between cells is not due to artifacts of the autoradiographic procedure but is, most likely, due to specific differences in cellular localization and/or cell cycle.

CHAPTER 6

COMPARISON OF AN IMMUNOCYTOCHEMICAL METHOD AND AUTORADIOGRAPHY FOR DETECTION OF THE OESTROGEN RECEPTOR

6.1 Introduction

Steroid hormone receptors can be measured directly through the application of antibodies that recognize the receptor protein: immunocytochemistry (Chapter 3). This chapter describes this direct visualization of the receptor molecule, using antibodies raised against the oestrogen receptor.

Several reports have been published on the production of polyclonal antibodies against the oestrogen receptor (Sofoff et al, 1969; Jungblut et al, 1970; Fox et al, 1976; Greene et al, 1977, 1979, 1980b; Coffer et al, 1980; Raam et al, 1981, 1983). To overcome problems of antibody specificity and the low titres of polyclonal anti-receptor antibodies, a few groups have produced monoclonal antibodies against oestrogen receptors (Greene et al, 1980a, 1982; Moncharmont et al, 1982). Greene et al (1984) established a library of about 10 monoclonal antibodies raised against the purified cytosolic oestradiol receptor complex from MCF-7 human breast cancer cells. The immuno(cyto)-chemical assays offer several advantages. It is suggested that the immuno(cyto)chemical assay is not influenced by 1) metabolism of the ligand during incubation, 2) in vitro proteolytic degradation of the receptor, 3) irreversible occupancy of the oestrogen receptor by endogenous ligand or 4) inability of binding of the steroid to the oestrogen receptor in its early stages of synthesis in vivo, which has not yet acquired its receptor capabilities. This latter point could be a disadvantage because it might contribute to false positive measurements. With the histochemical technique it is possible to detect a limited number of oestrogen receptor-positive cells in an otherwise oestrogen receptor-negative tissue. The immunocytochemical assay allows also the localization at the intranuclear level (Press et al, 1985). In contrast to the standard

biochemical assays it is possible to detect the "free" (not bound to oestradiol) receptor molecule. When compared to the autoradiographic technique, the immunocytochemical technique is less laborious to perform.

We have compared the immunocytochemical assay with the biochemical assay (Blankenstein et al, 1986), using cell lines and tissues with known oestrogen and progesterone receptor content. As a result from these studies, we have concluded that there is a good correlation between the biochemical and the immunocytochemical assay.

A disadvantage of the immuno(cyto)chemical assays may be that none of the monoclonal antibodies are generated against the steroid binding site. There might be a discrepancy between antigenic and steroid binding properties of the oestrogen receptor. Since the steroid binding assay and the immunocytochemical assay depend on different principles, it is possible to combine the two assays. We have therefore compared the steroid binding using autoradiographic techniques, and the immunocytochemical assay. These assays might mutually validate each other.

We have chosen the MCF-7 cell line as a model system because we observed in our autoradiographic studies that these MCF-7 cells are heterogeneous with respect to their oestrogen receptor content. Our results showed that there is a good agreement between the autoradiographic technique and the immunocytochemical assay (98%).

6.2 Results and discussion

6.2.1 Receptors in tissue

We have compared the immunocytochemical assay, using the monoclonal anti-oestrogen receptor antibody. H222 Spγ (a gift of Abbott Diagnostics, Chicago) and the biochemical assay, using (^{3}H)-E_2 as a ligand. Breast cancer specimens with known oestrogen and progesterone receptor content were used as an assay system. The specimens were sectioned at 6 μm and

processed for oestrogen receptor staining as recommended by the manufacturer. The results are summarized in Table 6.1. In breast cancer specimen G, containing 759 fmol ER/mg

Table 6.1 Results of biochemical and immunocytochemical assays of steroid hormone receptors in human breast cancer tissue and cell lines.

specimen	receptor content (fmol/mg protein)		specific immuno-chemical staining for nuclear ER
	ER	PgR	(% of cells)
breast cancer			
A	0	29	absent
B	17	871	5%
C	35	12	5-10%
D	61	0	30-40%
E1	105	28	40%
E2			5-10%
F	201	317	10-20%
G	759	3750	80%
cell-line			
MCF-7	75	90	68 ± 18%
PC-93	0	n.d.	absent
LNCaP-FGC	0	0	absent

Part of these results is published by Blankenstein et al. (1986).

protein, a large number of cell nuclei stained intensely for oestrogen receptor. A consistent result was observed in three separate experiments. Specimen C, with 35 fmol ER/mg protein, showed a moderate number of cell nuclei which were stained less intense than the cell nuclei in specimen G. In specimen B, with 17 fmol ER/mg protein, intense staining was confined to a limited part of the section, whereas in the remaining part only a few cell nuclei were faintly stained. This demonstrates that the sensitivity of the immunocytochemical assay for oestrogen receptor is sufficient to detect stained cells in breast cancer specimens containing as little as 17 fmol ER/mg cytosol protein. Specimen E, which was assayed at two occasions, showed different results. This observation might resemble the heterogeneity in a breast cancer specimen. The present data do not allow definite conclusions with respect

to the relation between quantitative oestrogen receptor levels and the percentage of immunocytochemically stained cell nuclei or the intensity of staining. To obtain more definite conclusions a larger series of samples will have to be examined. In this respect, the results of the studies of McCarty et al (1985) and King et al (1985) are of interest. They compared the immunocytochemical assay (using the monoclonal antibodies H222 Spγ or H226 Spγ) with the biochemical oestrogen receptor assays on a large sampling of human breast tumours (about 275 samples) and concluded that there is a significant association between the concentration of cytosolic oestrogen receptor and the presence or absence of nuclear staining. This proves that the immunocytochemical assay can be used for the detection of oestrogen receptors in breast cancer specimens.

McClellan et al (1984) showed that it is also possible to detect oestrogen receptors in the uterus, oviduct, cervix and vagina of macaque monkeys with the immunocytochemical technique. Non-target tissues did not contain cells which showed specific nuclear staining, supporting the specificity of this technique.

6.2.2 Receptors purified on sucrose density gradients

We have also incubated a radiolabelled cytosol of a breast specimen with either the anti-oestrogen receptor antibody or the control antibody. After centrifugation through a 10-30% sucrose gradient we observed that the incubation of the breast cancer specimen with the monoclonal oestrogen receptor antibody caused a marked shift in sedimentation value (4S to 8.5S). These shifts of peaks did not appear after incubation with the monoclonal control antibody (Figure 6.1). These results are consonant with the studies of Greene et al (1982).

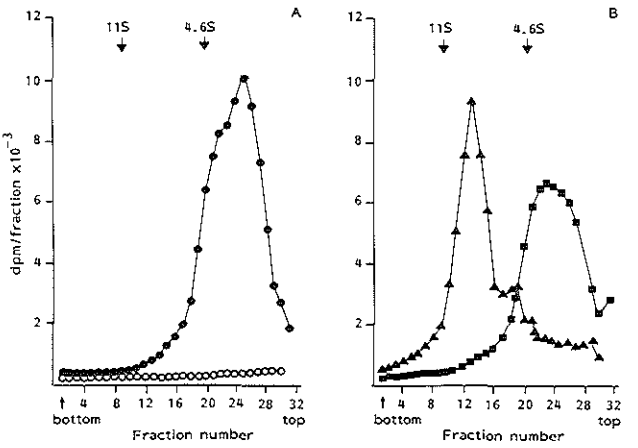


Figure 6.1. Sucrose gradient sedimentation profiles of a mammary tumour cytosol incubated with ^{3}H-E_2 (●-●) without or with (o o) a 200-fold molar excess of DES, panel A, and the same ^{3}H-E_2 labelled cytosol was incubated for 2 h with a monoclonal antibody (H222spy) raised against the oestrogen receptor (▲-▲) or a control antibody (■-■), panel B. The sucrose gradients (10-30% sucrose) contained 0.4 M KCl. Arrows indicate the sedimentation markers catalase (11S) and BSA (4.6S).

6.2.3 Receptors in tumour cells

The MCF-7 cells, containing 75 fmol ER/mg protein, also showed a heterogeneous staining pattern. Some cell nuclei were heavily stained whereas other cell nuclei were weakly or not stained (shown in appendix paper 4). PC-93 and LNCaP-FGC cells were shown to be oestrogen receptor negative by both the immunocytochemical and biochemical assay methods.

6.2.4 Comparison of autoradiography and immunocytochemistry in MCF-7 cells

Although the immunocytochemical and biochemical assays show a good correlation when used in breast cancer specimens and cell lines, it was still a matter of concern that none of the available monoclonal antibodies interact with the steroid binding site. The autoradiographic technique shows steroid binding whereas the immunocytochemical technique shows the receptor molecule. We have therefore applied the autoradiographic and immunocytochemical technique to the same specimens. The MCF-7 cell line was used as a model system.

First, studies were performed to obtain a reliable "combined assay" procedure. For that purpose MCF-7 cells were labelled with (^{125}I)-E_2, fixed in methanol for only 2 minutes, stained as recommended by the manufacturer and processed for autoradiography as described in Chapter 3. We observed weakly stained cell nuclei with some grains above the nuclei and most of the grains dispersed around the MCF-7 cells. This clearly demonstrated that the steroid is transported during the fixation and staining process and that the receptor molecules are retained in the cell nuclei. As a consequence, we have changed the sequence of the procedures in the assay. A comparable technique was used by Stumpf et al (1983) for combined immunocytochemistry and autoradiography of the progesterone receptor. The MCF-7 cells were incubated with (^{125}I)-E_2, thereafter the dry-mount autoradiography was performed, as described by Berns et al (Appendix paper 3) and after exposure the cells were mildly fixed and developed and the immunocytochemical assay was performed as recommended by the manufacturer. This technique revealed stained MCF-7 cell nuclei with grains above these nuclei. The photomicrographs are shown in appendix paper 4.

About 68±18% (mean±S.D.) of the MCF-7 cells showed a positive reaction for both combined immunocytochemical and autoradiographic techniques whereas 30±16% of the cells were not stained and also did not show grains above them. There was a good agreement (98%) between the presence of grains and specific staining of cell nuclei. We also observed that the intensity of staining and the amount of grains varied between the nuclei. It is not possible, with the present methodology, to quantitate or correlate the number of grains and peroxidase staining intensity, i.e. between steroid binding to the receptor molecule and the presence of the steroid hormone receptor molecule.

Using a mixture of two different monoclonal antibodies (D547 and D75 obtained from Greene) Kodama et al (1985) observed a comparable percentage of oestrogen receptor-negative MCF-7 cells. This heterogeneity of labelling and staining

observed between the cell nuclei might reflect biological variations in receptor levels related to changes in cell cycle, especially G1 and G2 phase (as described by Jakesz et al, 1984). This idea needs further investigation, and a technique using pulse-labelling of the MCF-7 cells with tritiated thymidine and staining with the monoclonal antibodies raised against the oestrogen receptor might reveal a possible relation between cell cycle and oestrogen receptor content.

About 2% of the MCF-7 cells were stained but did not retain the label above the cells. Several explanations exist for this observation: the detection level of the monoclonal antibody assay might be lower, the "stained" receptor molecule might be a proteolytic product without steroid binding capacity or the molecule, which does not have steroid binding capacity, might be immunologically identical to a receptor which can bind the steroid (a similar model has been described for the progesterone receptor by O'Malley et al, 1984). Our observations do not allow a clear conclusion. Further studies will be needed to elucidate this.

6.3 Conclusions

- The immunocytochemical assay offers a good possibility to discriminate between oestrogen receptor-positive and -negative cells in tissue sections and cell lines.
- Oestrogen receptor-positive cells show differences in staining intensity in the immunocytochemical assay, indicating that it is possible to distinguish between cells with high amounts and cells with lower amounts of oestrogen receptor.
- For breast cancer specimens and cell lines, the results of the immunocytochemical assay and the biochemical oestrogen receptor assay show a good correlation.
- The sensitivity of the immunocytochemical assay was found to be sufficient to detect staining in breast cancer specimens containing as little as 17 fmol ER/mg cytosol protein.
- A comparison of autoradiography with immunocytochemistry for

detection of oestrogen receptors in MCF-7 cells showed a good agreement (98%) between the presence of grains and specific nuclear immunostaining.

CHAPTER 7

STEROID HORMONE REGULATED CELL GROWTH AND THE RELEASE OF PROTEINS BY HORMONES IN HUMAN TUMOUR CELLS

7.1 Introduction

Differentiation and proliferation rates of human prostate and mammary carcinoma are dependent upon androgen or oestrogen stimuli respectively. The hormonal effects are mediated by intracellular receptors (Chapter 2). It is still unknown, however, how or via which mechanism(s) the growth of the tumour is regulated by steroid hormones, although the action of steroid hormones on target tissues has been studied for a long time. This chapter describes the steroid hormone regulated cell growth in vitro.

Several reports describe that oestrogens can stimulate and anti-oestrogens can suppress the cell growth of the MCF-7 cells (e.g. Lippman et al, 1976). In such a system oestrogens and anti-oestrogens were shown to regulate the expression of specific proteins (see Chapter 2). A summary is given in table 7.1.

Anti-oestrogens as such do not inhibit the synthesis of the 24 kD or the 52 kD protein but inhibit the induction of these

Table 7.1. Effect of oestrogens and anti-oestrogens on growth and induction of specific proteins in MCF-7 cells.

	Oestrogens (E2)	Anti-oestrogens (4OH Tamoxifen)	Literature reference
Growth	+	-	Lippman et al, 1976
PgR	+	+ *)	Horwitz et al, 1978
24 kD intracellular protein	+	-	Edwards et al, 1981
52 kD extracellular protein	+	-	Westley and Rochefort, 1980

*) Depending on the dose of 4-hydroxytamoxifen administered (Horwitz et al, 1978).

proteins by oestrogens (Edwards et al, 1981; Westley and Rochefort, 1980). In recent immunocyto- and immunohistochemical studies in both tumour and non-malignant tissues with monoclonal antibodies raised against these proteins (Adams et al, 1983; Garcia et al, 1984) it has been suggested that the 52 kD protein but not the 24 kD protein is a tumour specific protein (Ciocca et al, 1983; Ciocca and Dufau, 1984). A biologically active 52 kD protein has been purified from culture medium of oestradiol-treated cells by a two step procedure using concanavaline A-sepharose and IgG-sepharose chromatography. This 52 kD glycoprotein acts as an autocrine growth factor since conditioned medium from oestrogen stimulated MCF-7 cells as well as the purified glycoprotein stimulate the growth of the MCF-7 cells which have not been treated with oestrogens (Vignon et al, 1984, 1985, 1986).

In a comparable way androgen regulated growth factors may be involved in the growth of androgen responsive tumor cells. Although several human prostatic tumour cell lines are available, only the LNCaP (Lymph Node Carcinoma of the Prostate) cell line shows both hormone dependency and continuous growth in vitro. Another cell line PC-93, initiated from a human prostate carcinoma (van Steenbrugge et al, 1983), is not sensitive to androgens (van Steenbrugge, personal communication) and might serve as a control model.

In this chapter we describe culture conditions to study the effect of oestrogens on MCF-7 cell growth and protein release. A similar test system might be useful to study the androgen regulated prostate tumour cell growth. We have also studied the presence of receptors in and release of proteins by the human prostate cell lines LNCaP-FGC and PC-93.

7.2 Results and discussion

7.2.1 Oestrogen-regulated cell growth and release of proteins by MCF-7 cells

The effect of oestradiol on cell growth of MCF-7 cells

cultured in our laboratory was studied by Blankenstein et al (1985). They observed a maximal growth stimulation of 1.5-fold after addition of 1 nM oestradiol to stripped 10% fetal calf serum. This was a matter of great concern because Vignon et al (1983) observed a growth stimulation of 4.5-fold after administration of oestradiol added to stripped fetal calf serum to their MCF-7 cells. Leclerq (personal communication to Dr. Blankenstein) had suggested that the use of human serum instead of fetal calf serum might increase the stimulation factor. As a result Blankenstein and Foekens used stripped 10% human serum instead of 10% stripped fetal calf serum. They observed a 4-7 fold stimulation of MCF-7 cell growth with 1 nM or 30 pM oestradiol added to 10% stripped human serum.

We have studied the effects of oestradiol on the release of the 52 kD protein by the MCF-7 cells cultured in our laboratory. The effects of oestradiol without or with added 4-hydroxytamoxifen on the release of this protein were studied according to the protocol described by Westley and Rochefort (1980). After culturing the cells in 10% stripped fetal calf serum (without oestradiol), we observed a minor (^{35}S)-labelled 52 kD protein band. This result may indicate that our method of removing steroids from the serum by charcoal stripping is not sufficient or that other factors present in the fetal calf serum may stimulate the release of the 52 kD protein. In the presence of 1 nM oestradiol the secretion of the 52 kD protein was increased. 4-hydroxytamoxifen (1 μM) in combination with 1 nM oestradiol slightly reduced the release of the 52 kD protein. These results were not consonant with the results of Westley and Rochefort (1980), who observed an oestradiol induced release of the 52 kD protein. As described above, oestradiol added to stripped human serum in stead of fetal calf serum, significantly enhanced cell growth. Therefore, the effects of oestradiol on the release of the 52 kD protein using 10% stripped human serum instead of fetal calf serum were studied also. We observed a major 52 kD band after incubation with 1 nM oestradiol. This band was absent when the cells were incubated with vehicle

only. Moreover, we observed that the labelling of this 52 kD protein was reduced after incubation of the MCF-7 cells with both 1 μM 4-hydroxytamoxifen and 1 nM oestradiol.

In conclusion, the effects of oestradiol, especially after addition to human serum, on cell growth and on the release of the 52 kD protein together with the results of Vignon et al (1983, 1986) suggest that stimulation of cell growth and the release of a 52 kD protein are closely related.

7.2.2 Androgen-regulated cell growth of human prostate tumour cell lines: LNCaP-FGC and PC-93

We have studied the growth and the release of androgen-stimulated proteins by the human prostate tumour cell lines LNCaP-FGC and PC-93. These cell lines might be suitable for the investigation whether a similar auto- or paracrine growth situation (as described for the MCF-7 cells) is present for androgen-regulated growth of human prostate tumour cells in vitro.

The LNCaP-FGC cell line was made available to use by Dr. J. Horoszewicz (Buffalo) and the PC-93 was provided by Drs. van Steenbrugge (Department of Urology). Androgens stimulated the cell growth of the original LNCaP cell line and these cells also contained androgen receptors, both characteristics which are a prerequisite for our studies. We obtained a fast growing colony (FGC) from this cell line and we have checked whether androgens influenced the growth of this subline by studying the effect of anti-androgens and androgens on cell growth. The anti-androgen cyproterone acetate, added together with testosterone containing fetal calf serum suppressed the cell growth (appendix paper 5). This suggested that androgens might regulate the cell growth and we have therefore studied the growth of the LNCaP-FGC cells using charcoal dextran-treated fetal calf serum (to remove endogenous steroids: "stripped serum") with added androgens.

DHT, the natural ligand, increased the cell growth only at very high concentrations, because DHT is rapidly metabolized

(described in appendix paper 5). Therefore most of the studies were performed with the non-metabolizable androgen R1881. It is well known that R1881 can act also as a progestin via the progesterone receptor (Raynaud et al, 1977), but these cells do not contain progesterone receptors (appendix paper 5) so we have assumed that R1881 acts only as an androgen via the androgen receptor and can be used to study androgen-regulated cell growth in this cell line. We observed a biphasic dose-dependent stimulation of cell growth when R1881 was added at various concentrations (10 pM to 100 nM) in medium containing (15% v/v) stripped serum. The maximal growth stimulation factor is 2.5-fold (shown in appendix paper 5). The concentration of R1881 (0.1 nM) needed to maximally stimulate the LNCaP-FGC cell growth was lower than the concentration DHT (10 nM) to maximally stimulate the LNCaP cell growth in a similar extent (Horoszewicz, 1983). These results are consonant with the results of Schultz et al (1985) who observed also that a 100 fold lower amount of R1881 was sufficient to mimic the effects of 100 nM or 1 nM DHT. The rapid metabolism of DHT by the LNCaP cells might explain this discrepancy.

In conclusion: the LNCaP-FGC is considered to be hormone sensitive.

7.2.3 Steroid hormone receptors in the human prostate tumour cell lines: LNCaP-FGC and PC-93

Because it is well known that steroid hormones exert their effects via their receptors, we have studied the content of receptors for androgens, oestrogens and progestins in these cell lines. The results of the androgen receptor studies are summarized in Table 7.2. and have been extensively discussed for the LNCaP-FGC cell line in appendix paper 5, and for the PC-93 cell line in appendix paper 2.

As a result of these studies we have concluded that both the LNCaP-FGC and PC-93 cell line contain androgen receptors. The concentration of receptor in the LNCaP-FGC cell line is about 20 times higher when compared to that in the PC-93 cell

Table 7.2. Androgen receptor analysis, using sucrose density centrifugation in the human prostate tumour cell lines: LNCaP-FGC and PC-93.

androgen receptor	cell line			
	LNCaP-FGC		PC-93	
fmol/mg nuclear extract protein	1.679±558	(n=5)	82±42	(n=3)
sites/cell	17.000±2.500	(n=5)	925±530	(n=3)
S-value (0.4 M KCl)	4.55±0.20	(n=11)	5.07±0.15	(n=3)
and	2.83±0.24	(n=9)		

line. We observed two androgen receptor forms in the nuclear extracts of the LNCaP-FGC cells. These two forms could be of functional significance and were therefore further characterized. The results are summarized in Table 7.3. and discussed in appendix paper 5.

It is likely that the small receptor form (2.8S) in the nuclear extract is a proteolytic fragment from the large form (4.5S). This idea is supported by the following observations: 1) when a nuclear extract is left standing on ice, without protease inhibitors, we observed a shift from 4.5S to 2.8S, while the amount of steroid binding is not changed and 2) when the ACA34 column is run with a buffer containing bacitracine (a protease inhibitor), we observed mainly a protein with a calculated Mw of 91 kD, whereas when the ACA34 column is run

Table 7.3. Androgen receptor forms in the nuclear extract from LNCaP-FGC cells: Sedimentation (S) values, Stokes radii (Rs) and apparent molecular masses (Mr).

S-value	Rs (nM)	Mr (kD) *)
4.55 ± 0.20 (n=11)	4.7 - 4.8 (n=2)	90 - 92 (n=2)
2.83 ± 0.24 (n=9)	2.7 - 2.9 (n=2)	32 - 34 (n=2)

*) Mw: 4224 x (S20,w x Rs).

with a buffer containing insulin (which does not inhibit proteases), we observed mainly a protein with a calculated Mw of 33 kD. We have concluded that the 2.8S androgen receptor form is a proteolytic product from the 4.5S receptor form. Whether this proteolytic breakdown is of functional significance is not known and needs further investigation. In the cytosol of the LNCaP-FGC cells we observed about 23,000 androgen receptor sites/cell with a sedimentation value of 3.6S (in a high salt containing buffer with molybdate).

To assure that androgens exert their effects only via the androgen receptor, we have also studied oestrogen and progesterone receptors (appendix papers 1 and 5). We did observe an oestrogen binding protein with a K_d of 8.0 nM in the cytosol of the LNCaP-FGC cells. After sucrose gradient sedimentation (high salt, 0.4 M KCl), no specific binding was observed. Horoszowicz also observed an oestrogen binding protein (K_d of this protein was about 5.0 nM) in the cytosol of the LNCaP cells. The nature of this oestrogen binding "protein" is unknown, but according to the criteria listed in Chapter 2 this protein is not a receptor. Oestrogen and progesterone receptors were also not detectable in the nuclei of the LNCaP-FGC cells. Oestrogen receptors were also absent from the PC-93 cell line (appendix paper 1).

In conclusion, both the LNCaP-FGC and PC-93 cell line contain androgen receptors. The amount of receptor, however, in the LNCaP-FGC cells is about 20 times higher than in the PC-93 cells.

7.2.4 Androgen-released proteins by LNCaP-FGC and PC-93 cells

Finally, we studied the effects of androgens and anti-androgens on the production and release of proteins by LNCaP-FGC or PC-93 cells. Cells were grown in charcoal dextran-treated foetal calf serum (15% stripped serum) for four days and thereafter stimulated for 48 h with androgens (DHT or R1881) or with both androgens and anti-androgens, cyproterone acetate or RU 23908. Proteins were labelled for 6 h

after addition of 20 μCi (^{35}S)-methionine in 100 μl medium. Secreted proteins were analyzed on SDS-PAGE and fluorographed.

Most striking was the effect of androgens on the release of a 40 kD protein (appendix paper 5) from the LNCaP-FGC cell line. This protein was absent in the culture medium when untreated serum was substituted by charcoal dextran-treated serum, suggesting that the protein may be steroid-dependent. We have therefore added androgens to stripped serum. High concentrations of DHT (0.1-1 μM) and lower concentrations of R1881 (0.1-100 nM) restored the release of the 40 kD protein. Other steroids, oestrogens, progesterone and glucocorticoids did not have an effect. R5020, a synthetic progestin with androgenic activity (at high concentrations, Chalbos & Westley, 1984) also induced the release of the 40 kD protein at a concentration of 10 nM. The anti-androgens cyproterone acetate (added in a 100- 10,000 fold molar excess) or RU 23908 (added in a 1000 fold molar excess) in combination with androgens, did reduce the release of the Mw 40 kD protein (appendix paper 5). The low relative binding affinity of the anti-androgens can explain the high concentrations needed to counteract the androgenic effects. Cyproterone acetate had no effect on the 40 kD-released protein. We did not observe a specific release of a protein in the culture medium when PC-93 cells were stimulated with DHT (1 or 100 nM). The pattern of (^{35}S)-labelled protein bands did not differ from the controls when the PC-93 cells were cultured with 1 nM DHT with 1 μM cyproterone acetate.

The lack of response to androgens by the PC-93 cells might be explained by the low level of androgen receptors. It is difficult, however, to draw conclusions. Studies on metabolism of DHT by the PC-93 cells, the effects of the non-metabolizable androgen R1881 on PC-93 cell growth and on induction or release of specific proteins are needed to validate this explanation.

There are several reports on the release of proteins after stimulation with steroids. Of special interest is a report by Chalbos & Westley (1984), who described the release of specific progestin-induced proteins and of specific androgen-induced

proteins by the human breast cancer cell line, T47D. The androgen-released proteins by these cells had Mw's of 43 kD, 22 and 18 kD. We have only studied proteins with molecular weights above Mw 25 kD and we observed only a Mw 40 kD protein released from the LNCaP-FGC cells which is in the same Mw range as the Mw 43 kD protein released from the T47D cells. Edwards et al (1982) revealed with two dimensional gel electrophoresis in urine from prostate cancer patients a series of spots designed PCA-1 (prostate cancer antigen-1) with a Mw 40 kD, which was present only in urine of prostate cancer patients and also in prostate tissue. The latter findings suggest that the PCA-1 protein may be a non-secreted component of prostatic tissue that after transition to malignancy is released into the circulation.

Future studies with antibodies against the prostate cancer antigen, 40 kD, and the prostate specific antigen 35 kD, might reveal a possible relationship between the above mentioned 40 kD and 43 kD proteins.

7.3 Conclusions

- The human prostate cancer cell line LNCaP-FGC is androgen-sensitive and contains high amounts of androgen receptors.
- The human prostate cancer cell line PC-93 is insensitive to DHT and contains low amounts of androgen receptors.
- DHT did not affect the release of specific proteins by PC-93 cells.
- A 40 kD released protein disappears when the LNCaP-FGC cells are grown in medium containing steroid-free serum. Culture in the presence of DHT or R1881, restores the appearance of the 40 kD protein. Anti-androgens, which reduce cell growth, reduce the amount of the 40 kD released protein.
- The 40 kD protein might be involved in the regulation by androgens of malignant cell growth in the cancerous prostate.

CHAPTER 8

GENERAL DISCUSSION

The effect of "endocrine therapy" on the clinical improvement of patients with breast cancer has been shown to be related to the presence of oestrogen receptors in the cytosol of the tumour tissue. Significant levels of oestrogen receptors have been detected in about 60-70% of tumour tissues of patients with breast cancer and approximately 60-70% of these receptor positive tumours respond to endocrine therapy. In contrast only 5-10% of the oestrogen receptor negative tumours respond to endocrine therapy (Chapter 1). Studies on a possible relationship between the androgen receptor content in prostate cancer and the response to endocrine therapy revealed that the presence of nuclear androgen receptors, but not cytosol receptors, may be a useful parameter for the hormone dependency of prostate cancer (Chapter 1).

The correlations between the presence of receptors and the prognosis of the (development of the) malignancies, which were based mainly on biochemical receptor assays, are not perfect. Histochemical receptor assays, including cytofluorescence, autoradiography and immunocytochemistry, might enhance the accuracy of predicting the response to endocrine therapy. The latter methods will allow a more precise cell by cell analysis and may aid also in identifying specific target cells within heterogeneous tissues.

The purpose of the work reported in this thesis was to compare recently developed histochemical methods with biochemical methods for receptor analysis and to attempt to develop better biochemical markers for hormonal responsiveness of tumour cell growth.

8.1 Histochemistry of steroid hormone receptors

Biochemical assays for oestrogen receptors, which are routinely used for clinical purposes, yield quantitative

information about the amount of steroid bound to specific "receptor" proteins (according to EORTC standards). Such assays will give an impression about the capacity of the tissue to bind steroid, but cannot yield information about the cellular localization and origin of the receptor protein. In general large amounts of (protein containing) connective tissue are present in most mammary tumours and this provides a source of error. In addition, a relatively large amount of tissue (0.05-0.15 g; see table 8.1.) is required for an accurate estimation of binding. With the newer mammographic techniques breast tumours of less than 10 mm in diameter can be detected. However, such small amounts of cancerous tissues would not permit a biochemical assay of receptors. In this regard it was attractive to consider histo(cyto)chemical techniques, and several methods using cytochemistry, autoradiography and immunofluorescence have been developed (for assay principle see Chapter 3).

A useful histochemical assay should fulfil at least the following criteria: 1) it should distinguish between malignant and benign cells; 2) it should give quantitative information about the receptor content, as well as 3) the subcellular receptor distribution. In addition 4) the results of the histochemical assay should give a good correlation with the results of endocrine therapy.

Existing histochemical techniques have created considerable controversy, however, due to the conflicting results that were obtained. In an effort to elucidate the nature of the steroid binding revealed by histo(cyto)chemical methods, we have used a combination of biochemical and morphological techniques. The results from our studies as described in chapters 4, 5 and 6 and in the appendix papers, together with relevant data from the literature are summarized in table 8.1.

It is concluded from the results in (table 8.1.) that the cytofluorescence assays are not specific for detection of the oestrogen receptor, whereas the autoradiographic and immunocytochemical assays (with monoclonal antibodies raised against the human oestrogen receptor) are rather specific for

detection of the oestrogen receptor. These conclusions are based on the following observations:

1) The fluorescence assays that have been used depend on ligands which have a very low affinity (0.1-4.3%) for the receptor (Chapter 4). These assays reveal mainly cytoplasmic staining (Appendix papers 1 and 2) and when compared to the results obtained with the dextran-coated charcoal (DCC) assay a sensitivity of 75% and a specificity of only 50% is observed (table 8.1). In addition the results of the fluorescence assays do not correlate with other histochemical assays. Pertschuk et al (1982) have suggested that the fluorescent ligands bind to putative "type II" binding sites which might show a correlation with the amount of oestrogen receptors. These "type II" sites differ from the Type II binding described by Clark et al (1978) (see Chapter 2). We have observed, however, that oestrogen receptor-positive as well as oestrogen receptor-negative cell lines, when incubated with these ligands show fluorescence. Separation on PAGE of the binding proteins which bind the fluorescent ligands revealed that almost all fluorescent ligand is bound to albumin (Chapter 4). Hence, we have concluded that these low affinity ("type II") binding sites do not correlate with the presence of oestrogen receptors (Chapter 4). Based on these observations we have concluded that the fluorescent ligands used in our studies do not detect either oestrogen or androgen receptors.

2) The oestrogen receptor can be detected with both autoradiography (Appendix paper 3) and immunocytochemistry (Chapter 6). The autoradiographic technique depends on a functional feature of the receptor, i.e. the ability of the oestrogen receptor to bind oestradiol. The immunocytochemical method depends on the recognition of a specific antigenic site of the receptor by the antibody. Combined use of the autoradiographic and immunocytochemical technique offers the potential to validate the ability of both methods to detect the oestrogen receptor. When the two techniques were applied to the MCF-7 cell line an overall agreement of 98% was obtained (Appendix paper 4).

Table 8.1. Comparison of biochemical and histochemical oestrogen receptor assays.

Assay	Biochemical DCC/SDG	Cytofluor-rescence	Autoradio-graphy	Immunocyto-chemistry
"endpoint" of assay	label ^{3}H-/^{125}I-E_2	E_2-s-FITC/FA E_2-BSA-"/" 4-OH-Tamoxifen coumestrol	label ^{3}H-/^{125}I-E_2	monoclonal antibodies; e.g.H222spy/ H226spy
sample	cytosol/ nuclear extract	frozen section intact/frozen cells	frozen section intact cells	frozen section frozen cells
amount of tissue	50-150 mg	1-2 sections (4-6 m)	1-2 sections (4-6 m)	1-2 sections (4-6 m)
assay principle	steroid binding	steroid binding	steroid binding	antibody binding
duration (days)	2-3	1	>30 2 ^{3}H ^{125}I	1
R.B.A of the ligand	100%/75%	0.1-5%	100%/75%	-
is the assay quantitative	yes	no	no/semi	no/semi
receptor[1] detection	yes	no	yes	yes
receptor localization	no	-	yes	yes
correlation with biochemical assay in %, (ref.)	-	sens.[2] spec. 81 50 (1) 74 54 (1) 75 39 (4) 60 58 (7) 87 48 (7)	sens.[2] spec.[3] 91 78 (6) 100 - (8)	sens.[2] spec. 98 75 (5) 95 73 (1) 88 94 (2) 88 89 (3) 95 94 (3) 92 81 (9)
correlation with other histochemical assays	-	no[4]	yes[5] (98% with monoclonal antibody assay)	yes[5] (98% with autoradiography)

Notes:

1) The difference between the radioactive labelled steroid binding, measured in the absence and in the presence of a 100-fold molar excess of unlabelled steroid, is defined as the amount of "receptor".

2) Most reports describe a correlation between the histochemical and biochemical assays. Correlation, however, does not give a proper information on the sensitivity and specificity of the assay. We have therefore calculated the specificities (spec.) and sensitivities (sens.).

"Sensitivity" is defined as the proportion of true positives that are determined as positive by the test: TP/(TP + FN) x100%.

"Specificity" is defined as the proportion of true negatives that are determined as negative by the test: TN/(TN + FP) x100%.

TN, true negative; TP, true positive; FP, false positive; FN, false negative. (McCarty et al, 1980).

The percentages are calculated from the data described by: (1) Pertschuk et al, 1985; (2) McCarty et al, 1984; (3) McCarthy et al, 1985; (4) Fisher et al, 1982; (5) King et al, 1985; (6) Buell and Tremblay et al, 1985; (7) McCarthy et al, 1980; (8) Buell and Tremblay et al, 1983; (9) Shimada et al, 1985.

3) These results have been obtained with autoradiography using tritiated oestradiol.

4) In addition to our results, the data described by Parl et al (1984) and Stumpf et al (1984) have been used.

5) Obtained through the combined application of autoradiography, with (^{125}I)-oestradiol and immunocytochemistry using the monoclonal antibody H222spy (chapter 6). The results validate each other.

8.1.1 Autoradiography

Strobl et al (1979) have described that washing of a tissue with albumin efficiently decreases the non-specific binding but also resulted in a loss of some specifically bound oestradiol. Since we have used a comparable washing procedure in our radiographic studies, this might explain that the correlation between the immunocytochemical and autoradiographic study was 98%. The localization of non-covalently bound steroid (theoretically) without any redistribution and loss during tissue processing remains a goal to be achieved. The recently synthesized steroids, which bind covalently by (photo)affinity labelling (e.g. Katzenellenbogen et al, 1983; Horwitz et al, 1983 and Brinkmann et al, 1985) could offer a possibility to localize e.g. the oestrogen receptor after labelling with tamoxifen-azaridine on ultra thin frozen sections. The efficiency of this labelling is about 70% (Katzenellenbogen et

al, 1985). However, long exposure times are required when using this derivative for autoradiography since the specific activity of the tritium labelled compound is low.

The autoradiographic method described in this thesis (Chapter 5) makes use of iodinated steroids with a high affinity for the receptor. With this technique it is possible to detect the presence of receptors in different cells in a tissue. When compared with the DCC assay a high sensitivity (95%) and specificity (78%) was observed (Buell and Tremblay, 1983, 1985).

8.1.2 Immunocytochemistry

Immunocytochemical assays, using monoclonal antibodies raised against the oestrogen receptor, reveal predominantly nuclear staining (Chapter 6). Compared to the DCC assay the sensitivity of this techniques is 93% and the specificity 85% (combined results from table 8.1.). Oestrogen receptor positive tumours show a "patchwork" staining pattern and the intensity of nuclear staining of oestrogen receptor positive cells varies strongly. These observations might explain the discrepancy observed between the results of the immunocytochemical staining and those of the biochemical assay. In addition the monoclonal antibody assay detects also receptor-fragments which might not be able to bind steroids. To compare in a reliable way the results of the immunocytochemical assay and the DCC assay, especially in large tumours, multiple sections should be assayed with the immunocytochemical technique.

Few reports have been published on the correlation between the results of histochemical receptor assays (e.g. cytofluorescence and immunocytochemistry) and the response of the patients to endocrine therapy (Pertschuk et al, 1985; McCarty et al, 1984, 1985). If response to endocrine therapy is used as the ultimate criterion in evaluating assay reliability the immunocytochemical assay offers the best prospects. McCarthy et al (1985) studied a small group of 23 breast cancer patients,

from which fourteen patients responded to endocrine therapy. Biochemical assay of breast tissue revealed 10 oestrogen receptor positive patients (sensitivity 71%) and 7 oestrogen receptor negative patients (specificity 78%), whereas a semi-quantitative histochemical score (which included only the invasive tissue component) revealed 13 oestrogen receptor positive patients (sensitivity 93%) and 8 oestrogen receptor negative patients (corresponding to a specificity of 89%).

For the small group of patients studied, the immunocytochemical technique appears to offer a good parameter for predicting the clinical response of mammary cancer to endocrine therapy. However, until more information has been collected about the relationship between various patterns of immunocytochemical staining and clinical outcome, it seems prudent to consider the immunocytochemical assay not as a replacement for, but rather an adjunct to biochemical receptor assays.

Histochemical detection of androgen receptors could be useful also for the studies on the distribution of receptors in prostate tissues. Antibodies against the androgen receptor are not (yet) available, but Liao and Witte (1986) have reported recently on the presence of autoimmune anti-androgen-receptor antibodies in human serum. Further studies will be needed to validate these antibodies.

8.2 Steroid hormone regulated proteins and growth of tumour cells

The radiochemical measurement of oestrogen receptors in breast tumour cytosol is not a perfect parameter for predicting the growth response of oestrogen receptor positive breast cancers to endocrine therapy, since the correlation between the presence of receptor assays and response to endocrine therapy is only approximately 60% (Chapter 1). This poor correlation may be due to the fact that oestrogen receptors are involved only in the first steps of hormone action. In this respect several investigators have considered the possibility

that a better prediction might be obtained by measuring products of hormone action, such as mRNA or newly synthesized proteins (Chapter 2.7).

The progesterone receptor is an example of a protein synthesized under the influence of oestradiol and the progesterone receptor may be used as a marker for oestrogen (receptor) action. The use of progesterone receptor assays in addition to the biochemical oestrogen receptor assays in selecting patients for endocrine therapy has improved the response to endocrine therapy to about 70-80%. Patients with receptor rich tumours showed extended disease free periods and a better prognosis than patients with tumours containing little or no receptors (Clark et al, 1983). However anti-oestrogens, which inhibit cell-growth, can also induce the synthesis of progesterone receptors (Horwitz et al, 1978) which indicates that the presence of these receptors does not necessarily correlate with cell growth.

The exact mechanism of action of oestrogens and anti-oestrogens on cell growth is not known. Nevertheless, there is strong evidence that an oestrogen regulated protein such as the 52 kD released protein acts as a mitogen on human mammary tumour cells (Vignon et al, 1985, 1986; Rochefort et al, 1985). This 52 kD protein might serve as a biochemical marker for hormonal dependency of mammary tumour cell growth because: 1) this protein is present in mammary tumour cells which contain oestrogen receptors and whose growth is stimulated by oestrogens, 2) anti-oestrogens e.g. 4-hydroxytamoxifen, which prevent cell growth, also inhibit the synthesis of this protein. As a consequence it has been suggested that this 52 kD protein might be a better marker for oestrogen responsiveness of human breast cancer than the oestrogen receptor (Rochefort et al, 1984).

Based on the results obtained with the oestrogen stimulated growth of mammary tumour cells we have investigated the effects of androgens on the release of proteins by an androgen sensitive human tumour cell line, LNCaP-FCG (appendix paper 5).

We have observed an androgen induced release of a 40 kD protein. Other steroids did not cause the release of this protein. Horoszewicz et al (1983) reported on the release of prostate acid phosphatase after androgen stimulation and Schultz et al (1985) reported on the release of prostate acid phosphatase (50-54 kD) after androgen as well as oestradiol (high concentrations) addition to the LNCaP cells. These studies suggest that the release of prostatic acid phosphatase from the LNCaP-FGC cells might not be an androgen specific effect.

After incubation of the cultured cells with anti-androgens (such as cyproterone acetate and RU23908) this 40 kD protein was absent in the culture medium and cell growth was inhibited. This suggests that the release of the 40 kD released protein might be related to the growth rate of the androgen sensitive human prostate cell line, LNCaP-FGC and that the 40 kD protein might be involved in the regulation by androgens of malignant cell growth in the cancerous prostate. Finally it may be of interest to study similarities between this 40 kD and the prostate carcinogenic antigen, PCA (also 40 kD, described by Edwards et al, 1972) and the prostatic antigen, PA (35 kD, described by Wang et al, 1979).

8.3 Conclusions

From the results presented in this thesis and based on the additional evidence presented in this general discussion, we have concluded that:

- Both autoradiography and immunocytochemistry can be used in histochemical assays for a reliable detection of oestrogen receptors.
- The 40 kD protein, which is released by the cells of the human prostate cancer cell line LNCaP-FCG can be used as a marker for androgen responsiveness of these cells.

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SUMMARY

Steroid hormones trigger the induction of growth and/of the expression of specific gene(s) in a variety of target tissues. The hormone responsiveness of tissues and cells depends on the presence of a cellular protein termed the steroid hormone receptor.

It has been described that there is a variation in sensitivity of mammary and prostate tissues for oestrogens and androgens respectively. The success rate of the treatment of breast cancer patients with endocrine therapy is approximately two times higher if the tissue contains the specific receptor protein for the steroid hormone oestradiol. The presence of receptor for oestrogens and progestins appears to correlate with an even better prediction of the response of metastases on endocrine therapy. The correlations between the receptor content and response are, however, not perfect. Studies on prostatic carcinoma indicate that the nuclear androgen receptor concentration may be a useful parameter for the prediction of the effect of hormone treatment of prostate carcinoma. All biochemical receptor assays are performed on tissue homogenates of the histopathologically heterogeneous mammary and prostate carcinomas, but it is difficult to predict the response on basis of the receptor concentrations in a single biopsy. Histochemical receptor assays have been developed to circumvent these problems. One of the aims (described in chapter 1) of the present study was to evaluate these histochemical methods and to develop a reliable method for histochemical receptor detection in both mammary and prostate tissues.

Binding of a steroid hormone to the receptor is only the first step in the complex biochemical events which take place in the target cells. An assay which measures the steroid hormone induced growth response as such of a tumour may be more meaningful. Since steroids can influence cell proliferation and protein secretion, we have investigated also the effects of steroid hormones on tumour cell growth.

In chapter 2 the literature, dealing with the mechanism of

action of steroid hormones and the effect of steroids on cell growth, has been discussed.

Several histochemical methods for the visualization of steroid hormone receptors have been developed. These methods are are described in chapter 3 and have been evaluated using several criteria (chapter 4). These studies were performed with tumour cell lines and with normal and tumour tissues, all with biochemically characterized oestrogen and androgen receptor content.

The results of our studies show that receptors cannot be visualized with the low affinity fluorescent ligands (chapter 4 and appendix papers 1 and 2). With the autoradiographic techniques, however, including the rapid dry mount technique using (^{125}I)-oestradiol (appendix paper 3), it was possible to detect steroid hormone receptors in tumour cells and tissues (chapter 5). The histochemical method using monoclonal antibodies directed against the oestrogen receptor from MCF-7 cells, also allows the detection of this receptor in tumour cells and tissues (chapter 6). In MCF-7 cells the results obtained with combined autoradiographic technique and the immunocytochemical assay for oestrogen receptor showed a perfect correlation (appendix paper 4).

It is concluded (chapter 8) from the experiments described in this thesis that both the autoradiographic and the immunocytochemical assays as described offer reliable histochemical techniques for the detection of oestrogen receptors. Using these techniques it was possible to discriminate clearly between receptor-positive and receptor-negative cells in tumour tissues, and it was also possible to demonstrate differences in the amounts of receptor in the cells.

The effects of hormones on cell growth and the release of proteins by tumour cells are described in chapter 7 and appendix paper 5. These preliminary results show that the cells of the androgen responsive prostate tumour cell line (LNCaP-FGC) release a protein with a molecular mass of 40 kD only after stimulation with androgens. This 40 kD protein was not released after addition to the culture medium of

anti-androgens, which suppress cell growth. It is suggested that the steroid hormone regulated released proteins might provide additional information on the hormone dependent growth of the tumour.

SAMENVATTING

Een steroidhormoon, bijvoorbeeld oestradiol, progesteron of testosteron, oefent zijn werking op de zogenaamde doelwitcel uit door middel van binding aan bepaalde eiwitten in deze cel. Deze eiwitten, de steroidhormoon receptoren, hebben een hoge affiniteit voor het steroidhormoon. Binding van het hormoon aan de receptor resulteert in de beinvloeding van diverse biochemische processen hetgeen uiteindelijk leidt tot de synthese van bepaalde eiwitten of de groei van de doelwitcellen.

In de literatuur is beschreven dat bij mammacarcinoomcellen en prostaatcarcinoomcellen een variatie in de gevoeligheid voor respectievelijk oestrogenen of androgenen wordt gevonden. Ook is beschreven dat de kans op succes bij endocriene therapie van patienten met mammatumoren twee maal zo groot is indien het weefsel de receptor bevat voor het steroid hormoon oestradiol. Het gehalte aan cytoplasmatische receptoren voor oestrogenen én progestagenen is een nog betere parameter voor de prognose van een positief effect van diverse vormen van endocriene therapie op het ontstaan van uitzaaiingen (metastasen). De korrelaties tussen receptorgehaltes en respons zijn echter niet perfect. Bij de receptor-positieve mammatumoren is de kans op het (tijdelijk) wegblijven of verminderen van de ziekteverschijnselen (remissie) niet groter dan 70-80%. Bij prostaatcarcinoom worden de hormoonreceptoren meestal in de celkernen van de tumor bepaald. Deze bepaling heeft echter tot nu toe geen duidelijke voorspellende waarde bij een behandeling gericht op een verandering in het hormonale milieu.

Afgezien van problemen bij de kwantitatieve bepaling van deze labiele hormoonreceptoren, wordt de beperkte voorspellende waarde van de uitslag van een receptorenbepaling waarschijnlijk voornamelijk bepaald door de heterogeniteit van de tumoren en de aanwezigheid van zowel hormoon-afhankelijke, hormoon-gevoelige als hormoon-onafhankelijke cellen in de tumor. De veel gebruikte biochemische receptorbepalingen gaan uit van een homogenaat van de tumor en geven een redelijk betrouwbare kwantitatieve uitslag. Bij deze biochemische methode kan echter

geen rekening gehouden worden met boven genoemde heterogeniteit van de cellen in de tumor. Er is daarom geprobeerd om histochemische methodes te ontwikkelen voor het aantonen van receptoren in de cel.

De in dit proefschrift beschreven experimenten zijn uitgevoerd met het doel(zoals beschreven in hoodfstuk 1) om bestaande histochemische methoden voor de bepaling van oestrogeen- en androgeenreceptoren te evalueren, en om een bijdrage te leveren aan de ontwikkeling van een betrouwbare algemeen toepasbare methode voor histochemisch receptoronderzoek van zowel mamma- als prostaattumoren. De binding van een hormoon aan de receptor vormt slechts de eerste stap in de diverse gebeurtenissen die plaatsvinden in de doelwitcel, en een test welke direkt het effect van het hormoon op de groei van de tumor kan meten, zou waardevoller kunnen zijn. Omdat steroiden de celgroei en de secretie van specifieke eiwitten stimuleren, is ook geprobeerd om de effecten van steroiden op de celgroei te onderzoeken.

Hoofdstuk 2 geeft een overzicht van de relevante literatuur over de werking van steroidhormoon receptoren en de effecten van steroiden op celgroei.

Tot nu toe is er een drietal histochemische (cytochemische) receptor bepalingen beschreven welke gebruik maken van respectievelijk: 1) Fluorescerende verbindingen gekoppeld aan een steroid hormoon, 2) Autoradiografie met behulp van radioactieve steroiden en 3) Monoclonale antilichamen tegen de receptor. De principes van deze methoden zijn beschreven in hoofdstuk 3. Om de verschillende bepalingen te kunnen evalueren, hebben we gebruik gemaakt van de volgende kriteria: de verbinding moet 1) zuiver en stabiel zijn; 2) competeren met het natuurlijke ligand; 3) een hoge relatieve bindingsaffiniteit voor de receptor bezitten; 4) de binding moet verzadigbaar zijn; 5) de verbinding moet een lage niet specifieke binding vertonen en 6) er moet een goede correlatie zijn tussen kleuring en de biochemisch bepaalde receptor concentratie. Voor deze studie is naast tumor weefsel en normaal weefsel voornamelijk gebruik gemaakt van tumorcellijnen met bekende, op biochemische wijze

bepaalde, receptorgehaltes.

De eigenschappen van een aantal fluorescerende stoffen, met betrekking tot het zichtbaar maken van steroidreceptoren zijn door ons onderzocht bij oestrogeenreceptor- en/of androgeenreceptor-positieve cellijnen. Ter vergelijking werden receptor-negatieve cellijnen gebruikt. De verschillende gesynthetiseerde fluorescerende liganden die bij dit onderzoek gebruikt zijn, staan beschreven in appendix papers 1 en 2. In alle gevallen bleek dat de relatieve bindingsaffiniteit van het fluorescerende ligand voor de receptor zeer laag was (0.1-2%; hoofdstuk 4). Na kleuring met de fluorescerende oestrogene of androgene liganden werd de fluorescentie van de cellen bestudeerd. De fluorescentie intensiteit bleek niet te correleren met de aanwezigheid of de afwezigheid van receptoren, onafhankelijk van de gebruikte concentraties van de fluorescerende liganden. Hoewel het mogelijk zou zijn dat de fluorescerende liganden binden aan eiwitten met een lagere affiniteit en hogere capaciteit voor steroid hormonen bleek uit ons onderzoek ook dat deze mogelijke bindingsplaatsen niet gerelateerd zijn aan de aanwezigheid van de echte oestrogeen of androgeen receptor. Met behulp van niet-denaturerende gelelectroforese vonden wij dat de fluorescerende verbindingen met name goed bonden aan albumine. Daarom moet gekonkludeerd worden dat deze fluorescerende verbindingen geen onderscheid maken tussen oestrogeen- of androgeenreceptor-positieve en -negatieve cellen.

Ongeveer 20 jaar geleden werd aangetoond dat het mogelijk is om met behulp van autoradiografie specifiek receptoren aan te tonen. Helaas was de specifieke activiteit van het gebruikte tritium label zodanig laag, dat soms maanden nodig waren voor de belichting van de autoradiogrammen. Onlangs is een met radioactief jodium gelabeld oestradiol gesynthetiseerd, dat een 40 maal hogere specifieke activiteit bezit. Deze hoge specifieke activiteit maakt het in principe mogelijk de belichtingstijd aanzienlijk te verkorten, dat wil zeggen tot één à twee dagen. In dit proefschrift wordt de ontwikkeling van een autoradiografische methode met dit (^{125}I)-oestradiol

beschreven. Allereerst zijn de bindingseigenschappen van het 16α-(^{125}I)-oestradiol onderzocht (hoofdstuk 5 en appendix paper 3). De introductie van het jodiumatoom op de 16α plaats van het steroid zou de binding aan de receptor en het transport van het molecuul door de celmembraan kunnen beinvloeden. Uit de door ons gevonden resultaten blijkt, dat de bindingseigenschappen van het gejodeerde oestradiol aan oestrogeenreceptoren in kalfuteruscytosol en in MCF-7 cellen vergelijkbaar zijn met die van het tritium gelabelde oestradiol, het (^{125}I)-oestradiol diffundeert door de celmembraan en wordt gebonden door de receptor in de kern. De bindingsaffiniteit bedraagt 80% van de affiniteit van getritieerd oestradiol. Voor de autoradiografie met (^{125}I)-gelabelde oestradiol werd een specifieke wasprocedure ontwikkeld waarbij onder meer gebruik gemaakt werd van een antilichaam tegen oestradiol, waardoor de niet-specifieke achtergrond aanzienlijk verlaagd werd. Deze techniek biedt de mogelijkheid om binnen 48 uur specifiek receptoren aan te tonen zowel in cellen als in weefselcoupes.

Een andere zeer recente ontwikkeling is de mogelijkheid om de receptor te localiseren met specifieke monoclonale antilichamen die opgewekt zijn tegen de gezuiverde oestrogeenreceptor uit de humane mammatumor cellijn, MCF-7. Er is een goede correlatie gevonden tussen de resultaten van de biochemische receptor bepaling en de resultaten van deze histochemische methode om de receptor te lokaliseren zowel in weefselcoupes als in tumor cellen (hoofdstuk 6). De twee boven genoemde methoden (autoradiografie en het gebruik van monoclonale antilichamen) maken gebruik van een verschillend principe. In het ene geval wordt het gebonden hormoon aangetoond en in het andere geval wordt een gedeelte van het receptor eiwit aangetoond. Het is mogelijk de autoradiografische methode te kombineren met het gebruik van de monoclonale antilichamen (hoofdstuk 6 en appendix paper 4). Toepassing van beide technieken op eenzelfde celpreparaat (MCF-7 cellen) resulteerde in een goede correlatie tussen de resultaten van beide technieken (98% overeenstemming).

De effecten van hormonen op tumorcellen met betrekking tot

groei en secretie van eiwitten zijn beschreven in hoofdstuk 7 en appendix paper 5. Voorlopig kan geconcludeerd worden dat in hormoongevoelige prostaat tumorcellen (LNCaP-FGC) androgenen de secretie beinvloeden van een eiwit met een molecuulgewicht van 40 kD. Dit eiwit wordt niet uitgescheiden na toediening van anti-androgenen, die ook de celgroei in dit systeem remmen.

In hoofdstuk 8 zijn de resultaten van het onderzoek bediscussieerd. Wij hebben de konklusie getrokken dat de bestaande histochemische methodes met behulp van fluorescerende liganden geen receptor aantonen. De autoradiografische technieken, vooral bruikbaar met gejodeerd oestradiol, en de methode die gebruik maakt van monoclonale antilichamen tonen wel receptor aan. De resultaten van beide methodes geven onderling een goede correlatie. Het is dus mogelijk om met behulp van deze histochemische technieken de receptor-positieve cellen in het tumorweefsel op te sporen en de heterogeniteit in de verdeling van de receptor over de diverse cellen te bestuderen. Mogelijk kunnen in de naaste toekomst ook de onder invloed van hormonen gesecerneerde eiwitten aanvullende informatie verschaffen over de hormoongevoelige groei van de tumor.

ABBREVIATIONS

AR	androgen receptor
BSA	bovine serum albumin
CA	cyproterone acetate
CMO	carboxymethyloxime
CoAb	coupling antibody
(k)D	(kilo) Dalton
DES	diethylstilboestrol
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
E_2	oestradiol
EGF	epidermal growth factor
ER	oestrogen receptor
ERn	nuclear oestrogen receptor
ERICA	oestrogen receptor immunocytochemical assay
FA	fluoresceinamine
FGC	fast growing colony
FITC	fluoresceinisothiocyanate
γG	gamma globulin
GF	growth factor
GFR	growth factor receptor
K_d	dissociation constant
LNCaP	Lymph Node Carcinoma of the Prostate
M	Moles per litre
MCF	Michigan Cancer Foundation
MoAb	monoclonal antibodies
n	number of determinations
OV	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAP	phosphate acid phosphatase
PAPc	peroxidase anti-peroxidase complex
PCA	prostate carcinogenic antigen
PDGF	platelet derived growth factor
Pg	progesterone
PgR	progesterone receptor
R	receptor
(m)RNA	(messenger) ribonucleic acid
S	Svedberg unit
sd	standard deviation
SDS	sodium dodecylsulphate
SHBG	sex hormone binding globulin
t	transformation
v/v	volume/volume

NAWOORD

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 29 maart 1954 te Breda geboren. In 1971 behaalde zij het diploma HBS-B aan het Titus Brandsma College te Dordrecht. In hetzelfde jaar begon zij met de analistenopleiding aan het Van 't Hoff Instituut te Rotterdam (diploma HBO-A, klinische chemie in 1973, applicatiecursus HBO-B biochemie in 1974). In september 1974 werd begonnen met de studie Biologie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen B4 werd in juni 1978 behaald. Het doktoraalexamen, met als hoofdvakken Vergelijkende Endocrinologie en Experimentele Immunologie en bijvak Didaktiek van de Biologie, werd in april 1981 behaald.

Van 1 januari 1982 tot 1 januari 1986 was zij werkzaam bij de afdeling Biochemie II van de Erasmus Universiteit te Rotterdam. Gedurende deze periode werd het onderzoek verricht dat tot dit proefschrift heeft geleid. Dit werd mede mogelijk gemaakt door toekenning van een projectsubsidie door de Stichting Koningin Wilhelmina Fonds.

APPENDIX PAPER 1

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Report

Fluorescent ligands, used in histocytochemistry, do not discriminate between estrogen receptor-positive and receptor-negative human tumor cell lines

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Keywords: estrogen receptor, fluorescence histochemistry, steroid conjugates, tumor cell lines

Summary

A cell line containing estrogen receptors (MCF-7) and a cell line lacking estrogen receptors (PC-93) were used for a comparison of biochemical and histochemical procedures to detect estrogen receptors. We evaluated three different fluorescent estrogen derivatives: 17β-estradiol-6-carboxymethyloxime-bovine serum albumin-fluorescein isothiocyanate, 17β-estradiol-17-hemisuccinate-fluoresceinamine, and coumestrol. The main results were: 1. The relative binding affinities of these ligands for the estrogen receptor were between 0.1 and 2% of the affinity of estradiol. 2. Fluorescent staining of the cells showed no relation to the presence of estrogen receptors. 3. Staining was not suppressed with excess estradiol-17β, which is known to prevent binding of low affinity ligands to estrogen receptors. 4. Cells with intact membranes were not stained after treatment with the albumin-linked estrogen derivative; only cells with damaged cell membranes were stained. 5. Treatment of cells with 17β-estradiol-17-hemisuccinate-fluoresceinamine resulted in a fluorescent labeling of the cytoplasm in intact and artificially damaged cells. 6. Coumestrol caused only fluorescence of the cytoplasm in intact cells.

It is concluded that estrogen receptors cannot be detected with these low affinity ligands. Fluorescence of these cells is probably due to binding of the ligands to low affinity binding sites. The presence of these low affinity binding sites appears not to be related to the presence or absence of estrogen receptors and can therefore not be used to discriminate between estrogen receptor-positive and receptor-negative tumor cells.

Introduction

The presence of estrogen receptors in a breast tumor is a good indicator for a positive response of the tumor to endocrine therapy. The additional presence of progesterone receptors further enhances the probability of such a response (1). Until quite recently, the methods available for estimation of steroid receptors used the addition of the appropriate [^{3}H] steroid of the tumor cytosol proteins and the determination of the amount of tritium that became tightly bound to the receptors. Such techniques provide quantitative data and have been used for routine detection of solubilized steroid receptor proteins.

Methods for isolation and characterization of steroid-receptor complexes are time-consuming, they require special, expensive equipment and a

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minimum of 100 mg wet weight of tissue, and they do not reveal which or how many of the cells in the usually highly heterogeneous tumor specimen actually contain receptors. Hence, it would be attractive to develop histochemical methods for the visualization of the receptors. These methods might be faster and cheaper, they would permit more precise, cell-by-cell analysis and they might be applied to small amounts of tissue, or even aspirated cells.

Histochemical methods depend on either immunocytochemical localization of supposedly receptor-bound estradiol (2–5) or on direct visualization of receptor-bound steroid-fluorescein conjugates (6–11). A reliable localization of estrogen receptors would require that: 1) the fluorescent ligands have a high affinity for the receptor, to permit sensitive and specific detection of the small amounts of receptor; 2) nonspecific binding of the ligands to other cell constituents than the receptor is excluded; 3) a good correlation exists between the results of receptor estimation using biochemical and histochemical techniques.

The present report describes our attempts to use three fluorescent steroids (17β-estradiol-17-hemisuccinate-fluoresceinamine, 17β-estradiol-6-carboxymethyloxime-bovine serum albumin-fluorescein isothiocyanate, and coumestrol) in a histochemical assay for detection of estrogen receptors. For these studies we used model cell systems, with biochemically characterized estrogen receptor content and localization, i.e. the MCF-7 cell line with estrogen receptors and the PC-93 cell line without estrogen receptors. The results indicate that these ligands cannot be used to discriminate between estrogen receptor-positive and receptor-negative cells.

Materials and methods

Steroids

2, 4, 6, 7-[^{3}H]estradiol-17β (91.5 Ci/mmol) was obtained from New England Nuclear (Boston, Massachusetts, U.S.A.). Unlabeled steroids were purchased from Steraloids (Pawling, New York, U.S.A.). The radiochemical purity of the labeled estradiol-17β was verified by thin-layer chromatography.

Fluorescent ligands

Coumestrol [1-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofuran carboxylic acid lactone], a naturally occurring fluorescent plant estrogen, was a gift from Dr A.C. Notides (12).

17β-estradiol-6-carboxymethyloxime-bovine serum albumin-fluorescein-isothiocyanate (E_2-6CMO-BSA-FITC) was obtained from Zeus Scientific (Raritan, New Jersey, U.S.A.). This compound, first produced by Dr S.H. Lee (13), carries on the average 24–30 molecules of estradiol and 5–8 FITC residues on each BSA molecule.

17β-estradiol-17-hemisuccinate-fluoresceinamine was prepared from 17β-estradiol-17-hemisuccinate (E_2-HS) and fluoresceinamine. The hemisuccinate was synthesized according to Yellin (14). For each gram of E_2 used, 5 μCi [^{3}H]E_2 was added as a tracer. 17β-estradiol-17-hemisuccinate-fluoresceinamine was then synthesized using a modification of the method described by Joyce et al. (15). Stock solutions of the reactants were made up in dimethylformamide. 50 mg E_2-HS (40 mg/ml) was mixed with 50 mg fluoresceinamine (50 mg/ml) (Sigma), 50 mg dicyclohexylcarbodiimide (100 mg/ml) (Merck), and 12 ml acidified acetone. The mixture was stirred for 60 hr at 4° C in a dark room. The products were purified by preparative TLC, using the solvent chloroform:ethanol:water (54:12:1). The ligand was eluted and stored in ethanol at 4° C in the dark. A reference amount of 17β-estradiol-17-hemisuccinate-fluoresceinamine was kindly provided by Dr K. Griffiths (Tenovus Institute, Cardiff, Great Britain).

Cell culture

The MCF-7 human breast cancer cell line was provided by the Breast Cancer Animal and Human Tumor and Human Cell Culture Bank, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, U.S.A. A permanent human tumor cell line, PC-93, was provided by Mr G.J. van Steenbrugge, Erasmus University, Rotterdam.

This epithelial cell line, which originates from a prostate carcinoma, is hormone-independent.

The MCF-7 cells were cultured in medium RPMI-1640 with L-glutamine and 25 mM HEPES (Gibco Europe), containing 10 000 IU penicillin, 10 000 μg streptomycin (Seromed), 253 IU insulin (Organon), and 10% (v/v) fetal calf serum (FCS) (Boehringer Mannheim, F.R.G.). The PC-93 cells were cultured in Eagles Minimal Essential Medium (Gibco), containing 20 mM tricine (Sigma), nonessential amino acids (Gibco), 10 000 IU penicillin, 10 000 μg streptomycin and 10% (v/v) FCS. Cells were grown at 37° C under a humidified atmosphere of 5% CO_2 in air. Culture flasks, 75 cm^2, were supplied by Costar (Cambridge, Massachusetts, U.S.A.). The culture medium was changed twice a week.

Biochemical procedures

Incubation of cells

For each experiment 4 flasks with confluent cells were used. The cells were washed twice with phosphate-buffered saline (Dulbecco's, Gibco) and incubated for 1 hr at 37° C with 10^{-8} M tritium-labeled estradiol with or without 10^{-6} M diethylstilbestrol in RPMI-medium only. After incubation, the medium was discarded and the cells were processed for isolation of nuclear receptors, essentially as described by Mulder et al. (16). Briefly, the cells were removed from the flasks by scraping and were subsequently homogenized at 1° C in buffer A (10 mM Tris-HCl, 1.5 mM EDTA, and 1.5 mM dithiothreitol; pH 7.4 at 1° C). From this point all procedures were performed at 1° C. The homogenate was centrifuged at 700 g for 10 min, and the pellet was resuspended in buffer and washed 3 times with buffer A. (The buffer of the second wash contained additionally 0.2% Triton X-100.) The nuclear (700 g) pellet was extracted with buffer B (10 mM Tris-HCl, 0.4 M KCl, 1.5 mM EDTA, and 1.5 mM dithiothreitol; pH 8.5 at 1° C) for 60 min, followed by centrifugation of the nuclear extract at 105 000 g for 30 min.

Sucrose gradient centrifugation

For sucrose gradient centrifugation, nuclear extracts (200 μl, 0.1]-0.3 mg protein) were layered on 4.1 ml of a 5–20% (w/v) sucrose gradient, prepared in buffer B. After centrifugation in a Beckman L5-65B centrifuge at 1° C for 18 hr at 260 000 g using an SW-60 rotor, the bottom of the tube was pierced, 23–24 fractions were collected, and radioactivity was measured (17). γ-globulin (7.2 S), [^{14}C]bovine serum albumin (4.6 S), and ovalbumin (3.6 S) were used as sedimentation markers.

Protamine sulphate precipitation assay

Receptors were estimated essentially as described by Chamness et al. (18) with addition of 10 mM pyridoxal phosphate (final concentration) (19). The KCl concentration during precipitation was below 0.04 M.

Determination of the relative binding affinity

Uterine cytosol was prepared from the uteri of 2-day ovariectomized Wistar rats. The cytosol was incubated with 10 nM (^{3}H)estradiol-17β and increasing amounts of unlabeled competitor. After incubation for 16 hr at 4° C, the receptor content was estimated by the dextran-coated charcoal adsorption method (17). Nonspecific binding was determined in the presence of a 100-fold excess nonlabeled estradiol. Total binding was corrected for nonspecific binding resulting in specific binding (20).

Purification of albumin conjugate

For some experiments, free estradiol, estradiol-6-CMO, and free FITC were removed from 17β-estradiol-6-carboxymethyloxime-BSA-fluorescein isothiocyanate. One ml E_2-6-CMO-BSA-FITC (2×10^{-5} M) was layered on a Sephadex G-25 column (Pharmacia) and eluted with PBS-buffer. Fractions of 1 ml were collected. Fraction 4 contained 10^{-5} M E_2-6-CMO-BSA-FITC. This fraction was used in the relative binding affinity study. Free estradiol, estradiol-carboxymethyloxime, and FITC were retained by the column and were not eluted in the first 10 fractions of the eluate.

Protein determination

The protein content of cytosols and nuclear extracts was determined by the method of Bradford (21) with bovine serum albumin as standard.

Histochemical procedures

MCF-7 and PC-93 cells were washed twice with PBS-buffer in the culture flasks. After incubation for 5 min at 37° C with 1 ml of a 0.25% trypsin solution (Gibco), 5 ml culture medium was added and the cells were removed. After centrifugation for 3 min at 100 g, MCF-7 and PC-93 cells were subcultured in Falcon petri dishes (35 × 10 mm) in separate compartments for 2 days.

After 24 hr, culture medium was replaced in some dishes by medium containing 10% fetal calf serum which had been pre-extracted with dextran-coated charcoal to remove endogenous steroid hormones ('stripped medium'). After another 24 hr cells were used for histochemical staining.

Staining of intact cells

MCF-7 and PC-93 cells, cultured in normal or in stripped medium, were washed twice with PBS-buffer. Cells were incubated in 1 ml RPMI-medium, without FCS, with the ligand (10^{-5} M or 10^{-7} M final concentration), and in the presence or absence of 10^{-7} M estradiol, for 1 hr at 37° C. After incubation, the medium was removed and the cells were washed four times with PBS-buffer (buffer was changed every 15 min).

Staining of 'freeze-damaged' cells

MCF-7 and PC-93 cells, cultured either in normal culture medium or in stripped medium, were washed twice with PBS-buffer. Cells were 'freeze-damaged' according to Underwood et al. (22) with a slight modification. Briefly, RPMI-medium, with 2.6% (w/v) Ficoll (Pharmacia) to improve the final morphological appearance, was added to the cells in the petri dishes. Cells were immersed for 30 sec in liquid nitrogen and thawed at room temperature to simulate the freeze/thaw sequence in the preparation of frozen sections (22). The medium was aspirated and the resulting 'freeze-damaged' cells were air-dried for 1 hr in a refrigerator at 4° C. The dried cells were covered with 1 ml PBS-buffer containing the ligand in a final concentration 10^{-5} or 10^{-7} M, in the presence or absence of 10^{-7} M estradiol. Cells were stained for 2 hr at room temperature in a humid atmosphere. (Before covering with the ligand E_2-6-CMO-BSA-FITC, cells were rehydrated by covering them with a few drops of 2% BSA (w/v) in PBS, pH 7.4. After a few seconds the excess of BSA-buffer was wiped off (13).)

The staining solution was removed and the 'freeze-damaged' cells were washed with PBS-buffer for 1 hr. Buffer was changed every 15 min.

Microscopy

Cells were immediately examined under a fluorescence microscope (Leitz Orthoplan with epifluorescence, equipped with a 100 watt mercury bulb and an Orthomat). For coumestrol the cells were excited at 340 nm and viewed at 410 nm; for fluorescein, they were excited at 485 nm and viewed at 510 nm. The pattern and intensity of staining of the cells were evaluated, and recorded on Kodak Ectachrome 160 film.

Results

Estrogen receptor content of MCF-7 and PC-93 cells

Permanent tumor cell lines MCF-7 and PC-93 were investigated for estrogen receptor content in nuclei and cytoplasm. Figure 1 shows sucrose sedimentation profiles of nuclear extracts from MCF-7 cells and PC-93 cells. A peak of [^{3}H]estradiol sedimenting at 4.1 S was observed for MCF-7 cells. In nuclear extracts from PC-93 cells, no peak of [^{3}H]estradiol binding was observed. By protamine sulphate precipitation assay, 175 fmol receptor/mg protein was measured in nuclear extracts of MCF-7 cells while specific estradiol binding was not detectable in the nuclear extract of PC-93 cells.

Similar results were obtained for the cytoplasmic receptors by Scatchard binding analysis. Cytoplasmic estrogen receptor content in the MCF-7 cells was 65 and 84 fmol receptor/mg cytosol protein ($K_d = 0.31$ nM) in two separate experiments.

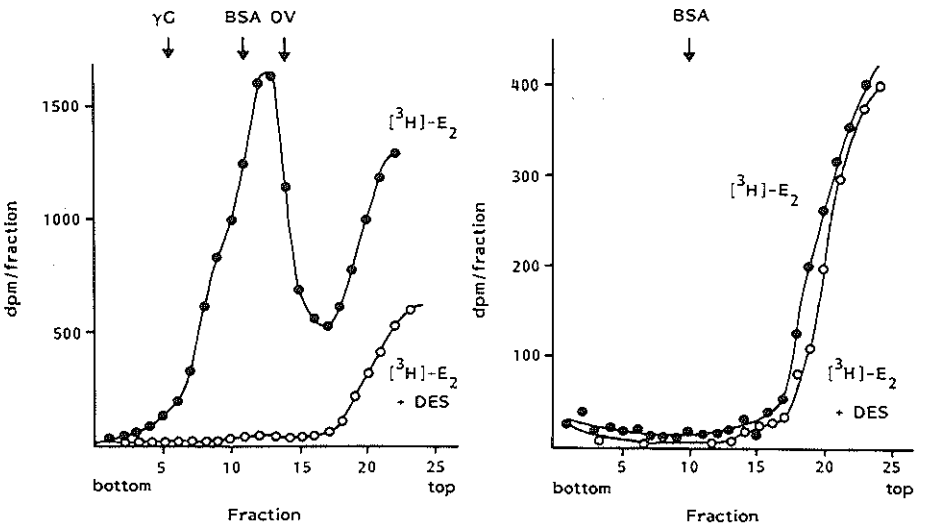

Fig. 1. Sucrose gradient sedimentation profiles of estrogen receptors extracted with 0.4 M KCl from nuclei of MCF-7 cells (left panel) and PC-93 cells (right panel). The cells were incubated for 1 hr at 37° C with 10 nM ^{3}H-estradiol (E_2) in the absence (●——●) or presence of a 100-fold excess of diethylstilbestrol (DES) (○——○). The sucrose gradients contained 0.4 M KCl. Gamma globulin (γ-G, 7.2 S), bovine serum albumin (BSA, 4.6 S), and ovalbumin (OV, 3.6 S) were used as sedimentation markers.

Estrogen receptors were absent from the PC-93 cells. In one experiment the cytoplasmic progesterone receptor content was 90 fmol receptor/mg cytosol protein ($K_d = 0.95$ nM) in the MCF-7 cells, while progesterone receptors were absent in the PC-93 cells.

Relative binding affinities of fluorescent ligands

Three fluorescent ligands were tested: E_2-6-CMO-BSA-FITC, E_2-HS-FA, and coumestrol (for structural formulas, see Fig. 2).

Uterine cytosol was incubated with 10 nM [^{3}H]estradiol and increasing amounts of unlabeled ligands. Percentage binding was measured by the charcoal technique. Relative affinities of the ligands for the estradiol-17β receptor in rat uterus are presented in Fig. 3. In Table 1 the calculated relative binding affinities are presented. The fluorescent ligands show low relative binding affinities of 0.1–5% compared to estradiol. Furthermore, when low molecular weight contaminants (e.g. free estradiol) were removed from the E_2-6-CMO-BSA-FITC preparation by gel chromatography on a Sephadex G-25 column, the value for the relative binding affinity of this fluorescent ligand decreased further to 1.8%, less than half of the value before chromatography.

Fluorescent staining of intact and 'freeze-damaged' MCF-7 and PC-93 cells

Intact cells were stained with 10^{-5} M and 10^{-7} M E_2-HS-FA, E_2-6-CMO-BSA-FITC, and cou-

Table 1. Relative binding affinities of fluorescent-labeled ligands.

Ligand	Relative binding affinity (%)
1. Estradiol	100
2. E_2-6-CMO-BSA-FITC	4.3
3. E_2-6-CMO-BSA-FITC (pretreated[a])	1.8
4. Coumestrol	1.4
5. E_2-HS-FA	0.1

[a] Partial purification of the ligand as described in Methods.

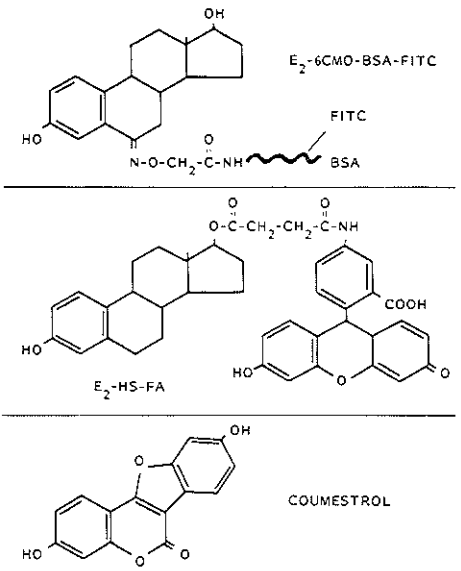

Fig. 2. Chemical formulas of the fluorescent ligands used for the staining of the cells: E_2-6-CMO-BSA-FITC (17β-estradiol-6-carboxymethyloxime-bovine serum albumin-fluorescein isothiocyanate), E_2-HS-FA (17β-estradiol-17-hemisuccinate-fluoresceinamine), and coumestrol.

mestrol as described in the Methods section. The results are shown in Table 2. No difference in fluorescent staining was observed between the estrogen receptor-positive cells, MCF-7, and the estrogen receptor-negative cells, PC-93, after addition of E_2-HS-FA (see Fig. 4a/b) or coumestrol. Addition of estradiol to the staining solution at a concentration of 10^{-7}M produced no decrease in intensity, and again no difference in fluorescent cytoplasmic staining between MCF-7 and PC-93 cells was observed. No fluorescence was observed with E_2-6-CMO-BSA-FITC, which is due to the impermeability of the cell membrane for the albumin derivative. Pretreatment of intact cells in estrogen-free medium for 24 hr prior to staining did not affect the fluorescence. Addition of FITC, the fluorescent moiety of the albumin estradiol complex, did result in a fluorescent staining of MCF-7 and PC-93 cells. This green stain differs in color shade from the E_2-6-CMO-BSA-FITC stain, which is bright apple-green. Addition of fluoresceinamine, the reagent used for synthesis of E_2-HS-FA, to the intact cells, did not reveal any fluorescence.

'Freeze-damaged' cells were also stained with 10^{-5} and 10^{-7}M E_2-HS-FA, E_2-6-CMO-BSA-FITC, and coumestrol. The results are shown in Table 2. Again no difference in fluorescent staining

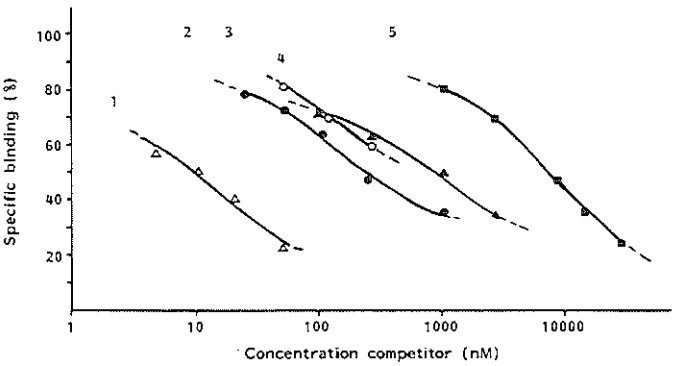

Fig. 3. Binding affinity of various ligands for the estradiol receptor from uterine tissue. Cytosol was prepared from uterus of mature ovariectomized rats, as described in Methods, and incubated with 10 nM [^{3}H]estradiol and increasing amounts of unlabeled ligands. Specific binding of [^{3}H]estradiol in the absence of the unlabeled competitors was set at 100%. Unlabeled ligands added were: (1) estradiol-17β; (2) E_2-6-CMO-BSA-FITC; (3) E_2-6-CMO-BSA-FITC after partial removal of free E_2 and/or E_2-6-CMO as described in Methods; (4) coumestrol; and (5) E_2-HS-FA. The parallel lines show the linear part of displacement curves plotted on a semi-log scale.

Table 2. Effect of different conditions on fluorescence of tumor cells (MCF-7 and PC-93).

Ligand (10^{-7})	Fluorescence			
	Intact cells		Damaged cells	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus
E_2-6-CMO-BSA-FITC	neg	neg	pos	pos
E_2-HS-FA	pos	neg	pos	neg
Coumestrol	pos	neg	neg	neg
FITC	pos[a]	pos[a]	pos[a]	pos[a]
FA	neg	neg	neg	neg

* Difference in staining intensities of MCF-7 and PC-93 cells: not observed
* Addition of excess estradiol (10^{-7} M) during incubation: no effect
* Incubation with 10^{-5} M fluorescent steroid instead of 10^{-7} M: increase in intensity

Intact and freeze-damaged MCF-7 and PC-93 cells were incubated with fluorescent ligands and prepared for fluorescence microscopy as described in Methods. Abbreviations of conjugated steroids are explained in the legend to Fig. 2:
pos: positive staining: apple green fluorescence for conjugated steroids, blue fluorescence for coumestrol;
neg: negative staining, no difference with background.
[a] Staining with FITC is considered as positive, although the intensity and color shade differs from staining with conjugated steroids.

was observed between MCF-7 and PC-93 cells. Both cell types stained with E_2-HS-FA showed a cytoplasmic fluorescence. When stained with E_2-6-CMO-BSA-FITC, both cell lines revealed cytoplasmic and nuclear fluorescence (see Fig. 4c/d), whereas with coumestrol, no fluorescence was observed at all. Addition of estradiol at a concentration of 10^{-7} M did not affect the staining pattern. With FITC and FA, results with damaged cells were the same as for the undamaged cells.

Discussion

The contradictory results with respect to the usefulness of fluorescent steroid conjugates for the detection of steroid hormone receptors in cells or tissues which have been published (2, 3, 5, 7, 8, 11, 13, 23–27), may have arisen from ill-defined tissue preparations and incubation techniques or impure preparations of steroid conjugates. Tissue sections contain variable preparations of intact cells, damaged cells, and dead cells, and during incubation decomposition and diffusion of proteins may occur. To circumvent these problems, the experiments in the present investigation were performed with preparations containing intact cultured cells only, or containing cells which were reproducibly damaged by a standard procedure. In addition, the preparations contained estrogen receptor-positive (MCF-7) cells or estrogen receptor-negative (PC-93) cells only, as demonstrated by biochemical analysis.

Three different fluorescent estrogenic ligands, E_2-6-CMO-BSA-FITC, E_2-HS-FA, and coumestrol were used for staining of the cells in our study. E_2-6-CMO-BSA-FITC, a fluorescent compound with several steroid molecules and fluorescent groups bound to one albumin molecule, has been used by Lee et al. (11, 13, 25) and selective staining of cells containing receptors is claimed. In E_2-HS-FA, the steroid and fluorescent molecules are linked directly through a short spacer. Joyce et al. (15) showed that this compound does not bind to classical estrogen receptors in thin sections of tumor tissue. With 1-(N)-fluoresceinyl-estrone thiosemicarbazone, a compound with a comparable structure, a good discrimination between estrogen receptor-positive and receptor-negative tissues has been reported (7, 27).

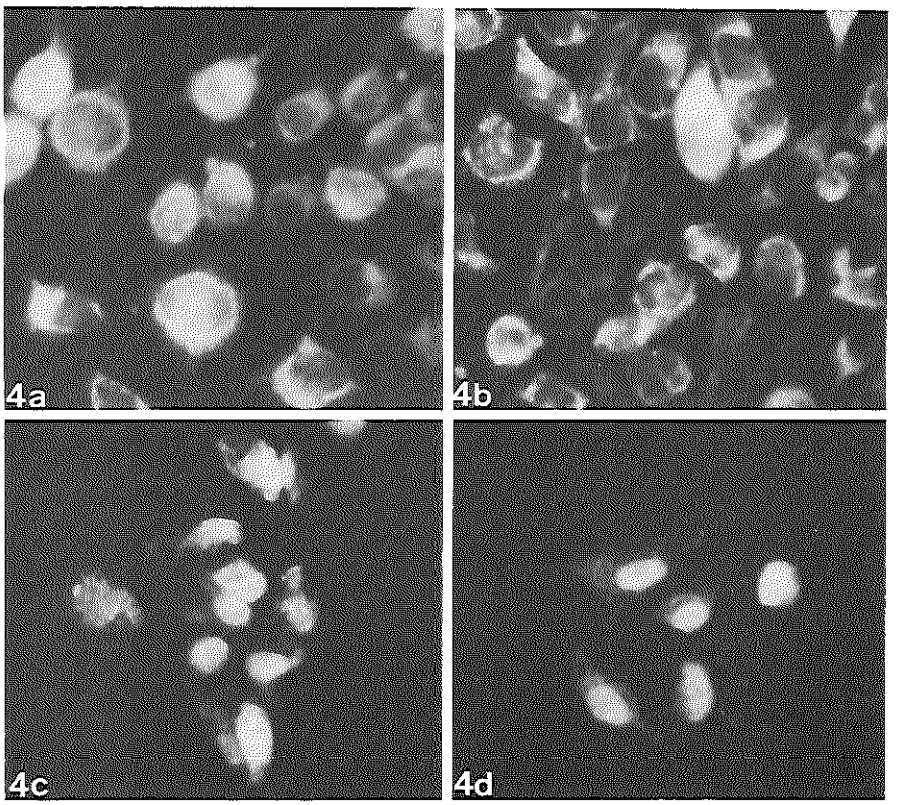

Fig 4. Intact MCF-7 cells (a) and PC-93 cells (b) stained with 10^{-5} M E_2-HS-FA for 1 hr, as described in Methods. 'Freeze-damaged' MCF-7 cells (c) and PC-93 cells (d) stained with 10^{-5} M E_2-6-CMO-BSA-FITC for 1 hr, as described in Methods. ×400.

The relative binding affinities of the fluorescent ligands for estrogen receptors were low, and one decreased further after partial purification (Table 1). For coumestrol the relative binding affinity found in this study was approximately 1%, a lower figure than previously observed (12, 28). These low binding affinities and the resulting high concentration of the compounds required to obtain a reasonable binding to the receptor, made it likely that nonspecific binding might occur. Such nonspecific binding could indeed be observed after incubation of intact cells of the two cell lines with E_2-HS-FA and coumestrol. Cytoplasmic fluorescence was independent of cell types and occurred even in the presence of an excess (10^{-7} M) estradiol. The absence of specific binding and the consequent disagreement with the biochemical data strongly indicate that the conjugates bind predominantly to nonspecific binding sites rather than to receptors.

Thin sections of tissues preparated for histochemical analysis are composed generally of a heterogeneity of intact and damaged cells. Therefore, the uptake of fluorescent compounds in damaged cells was also studied. Freeze-damaged cells were

prepared by a standard procedure essentially as described by Underwood et al. (22). Underwood concluded from these studies that the estrogen receptor might have been lost by diffusion. In our studies no differences in staining patterns were observed after damage to MCF-7 and PC-93 cells, stained with either E_2-HS-FA or E_2-6-CMO-BSA-FITC. The staining pattern was independent of the affinity of the conjugates, again illustrating the nonspecific character of the binding.

From the present study it can be concluded that the staining patterns observed for the fluorescent ligands E_2-HS-FA, E_2-6-CMO-BSA-FITC, and coumestrol, under different experimental conditions, showed no relation to the observed specific binding of estrogen receptors estimated by biochemical methods. Clark et al. (29) and Panko et al. (30) reported that 2–10 times more so-called 'type II binding sites' than true receptors may be present in breast carcinomas. However, standard fluorescence microscopy may not be sensitive enough to detect either the ca. 10 000 true receptor molecules in a cell, or the ca. 100 000 type II binding sites; some investigators working at the latter level have used electronic enhancement techniques (24). The fluorescence observed with the model cells used in the present study must therefore be due to binding of the ligands to low affinity binding sites present in a higher concentration than the type II binding sites. From the result of this study, it appears that the concentration of these low affinity binding sites, as estimated with fluorescent ligands, is not correlated with the concentration of true estrogen receptors and therefore cannot be used as a basis for discrimination between estrogen receptor-positive and receptor-negative cells. Future studies should concentrate both on the development of compounds with a high affinity for the receptor, and on intensified systems permitting the detection of low concentrations of fluorescent molecules (31).

Acknowledgements

The authors wish to thank Dr K. Griffiths (Tenovus Institute, Cardiff, UK) for valuable information on synthesis of 17β-estradiol-17-hemisuccinate-fluoresceinamine. The advice on isolation of receptors and assistance with the cell cultures of Mrs J. Bolt (Department of Urology) are greatly appreciated. Mr T.M. van Os provided valuable assistance in preparing the photomicrographs.

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APPENDIX PAPER 2

The Prostate 5:425–437 (1984)

Fluorescent Androgen Derivatives Do Not Discriminate Between Androgen Receptor-Positive and -Negative Human Tumor Cell Lines

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For the evaluation of histochemical procedures for detection of androgen receptors, three human tumor cell lines have been used: PC-93 and NHIK-3025, both biochemically characterized as androgen receptor-positive, and EB-33, biochemically characterized as androgen receptor-negative. The binding of three fluorescent ligands, testosterone-17β-hemisuccinate-bovine serum albumin-fluorescein isothiocyanate, testosterone-17β-hemisuccinate-fluoresceinamine, and 5α-dihydrotestosterone-17β-hemisuccinate-fluoresceinamine, to the cells was evaluated. The relative binding affinities of the ligands for the androgen receptors were low (less than 5% when compared to methyltrienolone). Treatment of the cells with the androgen-fluoresceinamine derivatives resulted in a fluorescent labeling of the cytoplasm in both intact and "freeze-damaged" cells of the three cell lines. This staining was independent of the presence of receptors. Nuclei were not stained.

Incubation of intact cells with the protein-linked conjugate did not result in significant cellular fluorescence. Only cells with damaged membranes showed a positive histochemical reaction, both in nucleus and cytoplasm, irrespective of the receptor content of the cells. The fluorescence intensity was not suppressed with excess 5α-dihydrotestosterone or methyltrienolone, which are known to prevent binding of low affinity ligands to androgen receptors.

From these results it is concluded that androgen receptors cannot be detected by these fluorescent ligands with low affinity for the receptor. The observed fluorescence of the cells is therefore due to binding of the ligands to other binding sites. The visualization/histochemical demonstration of these binding sites does not appear to be related to the presence of androgen receptors.

Key words: androgen, receptor, fluorescence, tumor cell lines, histocytochemistry

INTRODUCTION

Endocrine therapy has been the dominating form of treatment for advanced prostatic carcinoma for many years. In 75–80% of the patients with prostatic carcinoma, orchiectomy or estrogen therapy is efficient but relapses often occur. Although

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androgen receptor levels in human prostatic cytosols have been studied [1,2], the measurement of nuclear androgen receptors appeared to be most useful for the prediction of response and survival following hormonal therapy of man with metastatic prostate cancer [3,4].

Because prostate carcinoma is by no means a homogeneous histopathological entity and biochemical methods require a minimum of 25 mg of tissue [5], it is often difficult to predict response on the basis of a single biopsy. To circumvent these problems, histochemical methods have been developed [6–8]. Theoretically, these histochemical techniques for visualization of receptors (eg, with fluorescent ligands) would permit a more precise cell-by-cell analysis and might be applied to small amounts of tissue. For a sensitive and specific detection of small amounts of androgen receptors, however, several criteria should be fulfilled. First, the fluorescent ligands should have a high affinity for the receptor. In addition, nonspecific binding of the ligands to cell constituents other than the receptor should be excluded. As third criterion, a good correlation should exist between the results of receptor estimation using biochemical and histochemical techniques [9,10].

In this report, we describe the use of three fluorescent steroids (testosterone-17β-hemisuccinate-bovine serum albumin-fluorescein isothiocyanate, testosterone-17β-hemisuccinate-fluoresceinamine, and 5α-dihydrotestosterone-17β-hemisuccinate-fluoresceinamine) for detection and localization of androgen receptors in cells. For these studies, we used cell lines with biochemically characterized androgen receptors, ie, two cell lines, PC-93 and NHIK-3025, with different amounts of androgen receptor, and one cell line, EB-33, without androgen receptors.

The results indicate that these ligands cannot be used to discriminate between androgen receptor-positive and -negative cells.

MATERIALS AND METHODS

Steroids

[1,2,6,7-^{3}H]-Testosterone (SA: 93.9 Ci/mmol), 5α-dihydro-[1,2,4,5,6,7-^{3}H]-testosterone (SA: 145 Ci/mmol), 17β-hydroxy-17β-methyl-[^{3}H]-estra-4,9,11-trien-3-one (methyltrienolone, SA: 87 Ci/mmol), and unlabeled methyltrienolone were obtained from New England Nuclear, Dreieich, Federal Republic of Germany. Unlabeled testosterone (T), 5α-dihydrotestosterone (DHT), and the 17β-hemisuccinate derivatives were purchased from Steraloids, Pawling, NY. The radiochemical purity of the labeled steroids was verified by thin-layer chromatography (TLC).

Fluorescent Ligands

The preparation of testosterone-17β-hemisuccinate-bovine serum albumin-fluorescein isothiocyanate (T-BSA-FITC) was based on the mixed anhydride method used by Gaetjens and Pertschuk [11]. Briefly, testosterone-17β-hemisuccinate was coupled to bovine serum albumin in a molar ratio of 30:1 [12]. After incubation for 4 h at 4°C, the reaction mixture was dialyzed against 1 mM phosphate buffer, pH 7.4, containing 15 mM NaCl and centrifugated at 20,000g for 20 min to remove large aggregates. Gel chromatography was performed on AcA 44 (LKB, linear fractionation range 10–150K daltons, column size 90 × 1.5 cm) and the 70K dalton steroid-protein conjugate was obtained, dialyzed to distilled water, and lyophylized.

Testosterone-bovine serum albumin conjugate (T-BSA; 20 mg) was reacted with 2 mg of FITC isomer (Eastman) in 5 ml 0.1 M carbonate buffer, pH 9.5. After overnight incubation at 4°C, the free FITC was removed by Sephadex G-25 filtration. The conjugate carried per mole BSA on the average 13 moles of testosterone, as determined according to Erlanger et al [12], and 5 to 6 FITC residues, quantified as described by Steinbach and von Mayersbach [13].

Testosterone-17β-hemisuccinate-fluoresceinamine (T-HS-FA) was prepared according to the mixed anhydride method [12]. Briefly, to 20 μmol of testosterone-17β-hemisuccinate dissolved in 100 μl of dimethylformamide, 20 μmol of tributylamine and 20 μl of isobutylchloroformate were added. After incubation for 30 min at 4°C, 20 μmol of fluoresceinamine isomer I were added, and the reaction could proceed for 60 min at 4°C in the dark. The fluoresceinated testosterone was purified on preparative silicagel plates (Merck) using the solvent system chloroform:methanol: ammonia:water = 95:54:5.5:5.5 (v/v).

Dihydrotestosterone-17-β-hemisuccinate (DHT-HS-FA) was synthesized according to Joyce et al [14] and purified by preparative TLC using the solvent system chloroform:ethanol:water = 54:12:1 (v/v). Reference amounts of T-HS-FA and DHT-HS-FA were kindly provided by Dr. K. Griffiths (Tenovus Institute, Cardiff, UK). The structural formulas of the fluorescent ligands used in this study are given in Figure 1.

Relative Binding Affinities of the Fluorescent Ligands

The relative binding affinities of the fluorescent ligands for the androgen receptors in the cytosol of the prostate from a castrated rat were determined according to Bonne and Raynaud [15]. To a series of prostate cytosol samples, a constant concentration of labeled methyltrienolone and increasing concentrations of unlabeled competing fluorescent ligands were added, and binding of label was measured with a charcoal assay.

The relative binding affinities were calculated as the ratio of concentration of methyltrienolone and the fluorescent ligand required to reduce labeled methyltrienolone specific binding by 50%. The relative binding affinities, for the androgen receptor, of DHT-17β-HS-FA, T-17β-HS-FA, and T-17β-HS-BSA-FITC were 4%, 0.1%, and 0.1%, respectively, when compared to methyltrienolone (100%).

Cell Culture

The permanent human tumor cell line, PC-93, and the EB-33 cell line, both initiated from a human prostate adenocarcinoma and shown to be hormone-independent, were provided by the Department of Urology, Erasmus University, Rotterdam [16,17]. The cell line NHIK-3025 was derived from an early stage of a carcinoma of the human uterine cervix and contains androgen and corticoid receptors, but no estrogen and progesterone receptors [18,19]. The cells were cultured at 37°C in Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY) containing 10,000 IU penicillin, 10,000 μg streptomycin, and 10% (v/v) fetal calf serum (Gibco), in a humidified atmosphere of 5% CO_2 in air. Culture flasks (75 cm^2) were obtained from Costar (Cambridge, MA). The cells were trypsinized once a week, and the culture medium was changed twice a week.

Biochemical Procedures

Receptor isolation. For each experiment, four flasks with confluent cells (1–2 × 10^7 cells) were used. For isolation of nuclear receptor, the cells were washed twice

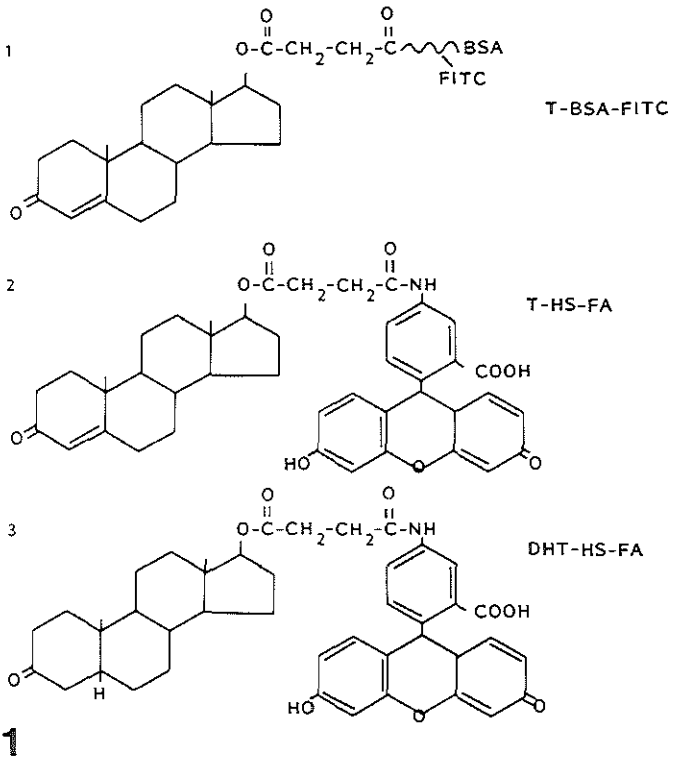

Fig. 1. Structural formulas of the fluorescent ligands used for the staining of the cells. 1) T-BSA-FITC, testosterone-17β-hemisuccinate-bovine serum albumin-fluorescein isothiocyanate; 2) T-HS-FA, testosterone-17β-hemisuccinate-fluoresceinamine; 3) DHT-HS-FA, 5α-dihydrotestosterone-17β-hemisuccinate-fluoresceinamine.

with Dulbecco's phosphate-buffered saline (PBS buffer; Gibco) and incubated for 1 hr at 37°C in MEM with 20 nM [^{3}H]-labeled testosterone with or without a 100-fold molar excess of unlabeled testosterone, DHT of methyltrienolone. After incubation, the medium was discarded, and the cells were processed for isolation of nuclear receptors as described previously for NHIK-3025 cells [19]. For estimation of cytoplasmic receptor content, the cells were collected in a small volume, homogenized, and the 100,000g supernatant of this homogenate was labeled with [^{3}H]-methyltrienolone or [^{3}H]-DHT, as described before [19].

Sucrose gradient centrifugation. For sucrose gradient centrifugation, nuclear extracts (200 μl, 0.2 to 0.3 mg protein) were layered on 4.1 ml of a 5–20% (w/v) sucrose gradient, prepared in a buffer (10 mM Tris-HCl buffer, with 1.5 mM EDTA; 1.5 mM dithiothreitol and 10% glycerol (v/v), pH 8.5 (1°C); with 0.5 M KCl). After centrifugation in a Beckman L5-65B centrifuge at 1°C for 23 hr at 300,000g_{av} using a Beckman SW-60 rotor, the bottom of the tube was pierced, 24 to 25 fractions were

collected and radioactivity was measured [20]. γ-Globulin (7.2 S), bovine serum albumin (4.6 S) and ovalbumin (3.6 S) were used as sedimentation markers.

Receptor assay. Receptors were estimated essentially as described by Chamness et al [21] by precipitation of protamine sulphate after addition of 10 mM pyridoxal phosphate (final concentration) [22]. The KCl concentration during precipitation was less than 0.05 M KCl.

DNA determination. The DNA content of the nuclear receptor preparations was determined by the method of Giles and Myers [23] with calf thymus DNA (Sigma, St. Louis, MO) as a standard.

Histochemical Procedures

Preparation of cells. PC-93, NHIK-3025, and EB-33 cells were washed twice with PBS buffer in the culture flasks. After incubation for 5 min at 37°C with 1 ml of a 0.25% trypsin solution (Gibco), 5 ml culture medium was added and the cells were harvested. After centrifugation for 5 min at 100g, the supernatant was discarded, the cells were suspended in fresh medium, seeded in Falcon petri dishes (35 × 10 mm) in separate compartments, and cultured for 2 days.

Staining of intact cells. The cells were washed twice with PBS buffer and incubated in 1 ml medium, without FCS, containing the ligand (10^{-5}, 10^{-7}, or 10^{-9} M final concentration) and either in the presence or absence of 10^{-7} M DHT or methyltrienolone for 1 hr at 37°C. After incubation, the medium was removed, and the cells were washed four times with PBS buffer (buffer was changed every 15 min).

Staining of "freeze-damaged" cells. The cells were washed twice with PBS buffer. Cells were "freeze-damaged" according to Underwood et al [24] with a slight modification. Briefly, MEM with 2.6% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) was added to the cells in the petri dishes to improve the final morphological appearance. Cells were immersed for 30 sec in liquid nitrogen and thawed at room temperature to simulate the freeze/thaw sequence in the preparation of frozen sections [24]. The medium was aspirated and the resulting "freeze-damaged" cells were air-dried for 1 hr in a refrigerator at 4°C. The dried cells were covered with 1 ml PBS buffer containing the ligand in a final concentration of 10^{-5} or 10^{-7} M, either in the presence or absence of 10^{-7} M DHT or methyltrienolone. Cells were stained for 2 hr at room temperature in a humid atmosphere. (Before covering with the ligand T-BSA-FITC, cells were rehydrated by covering them with a few drops of 2% BSA (w/v) in PBS, pH 7.4. After a few seconds the excess of BSA buffer was wiped off [25].) The staining solution was removed and the "freeze-damaged" cells were washed with PBS buffer for 1 hr. Buffer was changed every 15 min.

Microscopy. Cells were immediately examined under a fluorescence microscope (Leitz Orthoplan with epifluorescence: equipped with a 100 W mercury bulb and an Orthomat-camera). Fluorescence of the cells was studied with an excitation wavelength of 485 nm and an emission wavelength of 510 nm. The pattern and intensity of staining of the cells was evaluated and recorded on Kodak Ectachrome 160 film.

RESULTS

Androgen Receptor Content

The androgen receptor content in the nuclear extracts of the tumor cell lines PC-93, NHIK-3025, and EB-33 was determined. In Figure 2, the sucrose gradient

sedimentation profiles obtained after incubation of the cells with [^{3}H]-testosterone are shown. A peak of radioactivity sedimenting at 4.6–4.8 S was observed for both PC-93 and NHIK-3025 cells. A 100-fold molar excess of either testosterone, DHT, or methyltrienolone caused a complete displacement of radioactive label, indicating the limited capacity of this binding system. No incorporation of label was observed in the nuclear extracts from EB-33 cells, incubated either with [^{3}H]-testosterone, [^{3}H]-DHT, or [^{3}H]-methyltrienolone. The receptor content of the nuclear extracts was also measured with a protamine-sulphate precipitation assay. Amounts of 49, 57, and 8 fmol receptor/mg DNA were measured for PC-93, NHIK-3025, and EB-33 cells, respectively. The presence of only limited amounts of labeled steroid receptor complexes in the nuclear extracts of EB-33 cells could be due to a defective nuclear translocation mechanism of the androgen receptor in these cells. Therefore, we have also measured the androgen receptor content in the cytosol fraction of the EB-33 cells, and again very low amounts of androgen receptor were measured (less than 2 fmol receptor/mg cytosol protein).

From these results, we conclude that the PC-93 and NHIK-3025 cells are androgen receptor-positive, and the EB-33 cells are androgen receptor-negative (see also Table II).

Staining With Fluorescent Ligands

Intact cells were incubated with 10^{-5}, 10^{-7}, and 10^{-9} M T-BSA-FITC, T-HS-FA, and DHT-HS-FA as described in Materials and Methods. The results are shown in Tables I and II. No difference in fluorescent staining was observed between the

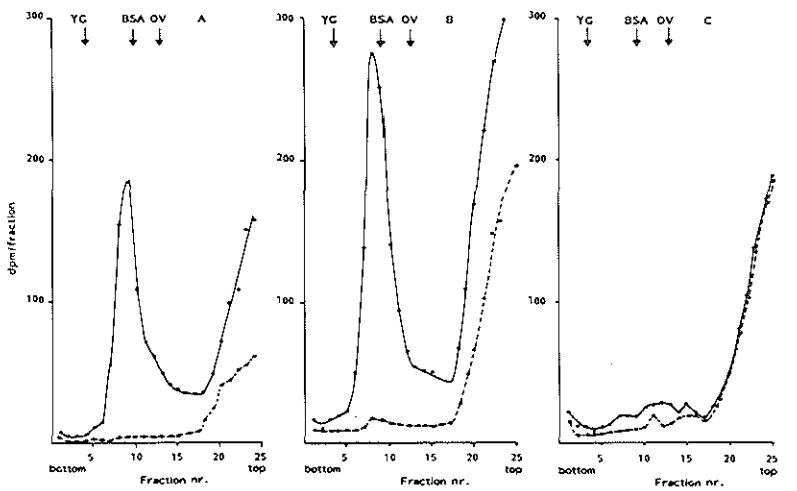

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Fig. 2. Sucrose gradient sedimentation profiles of nuclear extracts from PC-93 cells (A), NHIK-3025 cells (B), and EB-33 cells (C). The cells were incubated for 1 hr at 37°C with 20 nM (^{3}H)-testosterone (T) in the absence (•——•) or presence (broken line) of a 100-fold excess of testosterone. The sucrose gradients contained 0.5 M KCl. Gamma globulin (γG, 7.2 S), bovine serum albumin (BSA, 4.6 S), and ovalbumin (OV, 3.6 S) were used as sedimentation markers.

three different cell lines after addition of T-HS-FA or DHT-HS-FA (Fig. 3). Simultaneous incubation of the cells with both the fluorescent ligand and 10^{-7} M DHT or methyltrienolone did not result in a decrease of the staining intensity, and again no difference in fluorescent staining of the cellular cytoplasm between the androgen receptor-positive and -negative cell lines was found. The intensity of the fluorescence after incubation with T-BSA-FITC was low, and the color differed markedly from

TABLE I. Localization of Fluorescence in PC-93, NHIK-3025, and EB-33 Tumor Cells Incubated With Fluorescent Androgen Derivatives FITC or FA

Ligand (10^{-7} M)	Fluorescence of: Intact cells — Cytoplasm	Intact cells — Nucleus	"Freeze-damaged" cells — Cytoplasm	"Freeze-damaged" cells — Nucleus
T-BSA-FITC	DG	DG	Pos	Pos
T-HS-FA	Pos	Neg	Pos	Neg
DHT-HS-FA	Pos	Neg	Pos	Neg
FITC	DG	DG	DG	DG
FA	Neg	Neg	Neg	Neg

Pos = positive staining, apple green fluorescence for the conjugated steroid; Neg = negative staining, no difference with the autofluorescence of the cells; DG = dark green fluorescence, color shade and intensity differs from preparations indicated as positive.

TABLE II. Concentration of Androgen Receptors in Nuclear Extracts and Characteristics of Fluorescence Observed After Incubation of PC-93, NHIK-3025, and EB-33 Tumor Cells With Fluorescent Androgen Derivatives

Parameter	Cell line: PC-93	NHIK-3025	EB-33
Nuclear androgen receptor content (fmol/mg protein)[a] (gradient assay)	18	16	2
Protamine sulphate precipitation assay (fmol/mg DNA)	49	57	8
Receptor classification	positive	positive	negative
Intensity of fluorescence relative to autofluorescence[b] with:			
10^{-5} M steroid derivative	++++	++++	++++
10^{-7} M steroid derivative	++	++	++
10^{-9} M steroid derivative	−	−	−
Displacement of fluorescence by the native androgen receptor ligand (DHT or methyltrienolone), concentration 10^{-7} M	none	none	none

[a]The receptor content is expressed as fmol/mg protein in the nuclear extract.
[b]Cytoplasmic fluorescence of "freeze-damaged" cells. This fluorescence is independent of the structure of the fluorescent compound used; see also Table I.

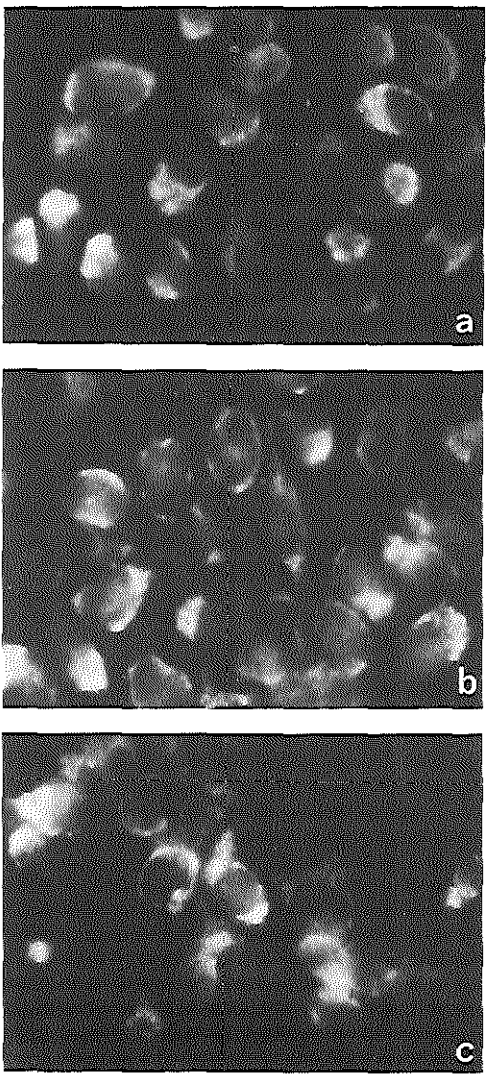

Fig. 3. Intact PC-93 (a), NHIK-3025 (b), and EB-33 (c) cells stained with 10^{-5} M of T-HS-FA for 1 hr as described in Materials and Methods. Only cytoplasmic staining is observed in all three cell lines. ×400.

the "bright apple green" fluorescence observed after incubation of the "freeze-damaged" cells with T-BSA-FITC (see Table II).

Application of unconjugated FITC instead of the T-BSA-FITC conjugate resulted in a similar fluorescent staining of the cells. Therefore, staining of the intact cells after incubation with T-BSA-FITC may be due to contaminating unconjugated FITC. Addition of fluoresceinamine, the reagent used for synthesis of T-HS-FA and DHT-HS-FA to intact cells did not reveal any fluorescence.

"Freeze-damaged" cells also were incubated with 10^{-5}, 10^{-7}, and 10^{-9} M of each of the ligands T-BSA-FITC, T-HS-FA, and DHT-HS-FA. The results are shown in Tables I and II. No difference in fluorescent staining was observed between the three cell lines. The cells incubated with T-HS-FA or DHT-HS-FA showed only a cytoplasmic fluorescence, whereas the three cell lines revealed more nuclear than cytoplasmic fluorescence after incubation with T-BSA-FITC (see Fig. 4). Simultaneous incubation of the cells with both the fluorescent ligands and 10^{-7} M DHT or methyltrienolone did not reduce fluorescence. With unconjugated FITC and FA, the results for the damaged cells were the same as for the intact cells.

DISCUSSION

Fluorescent labeled androgens could be of great value as reagents for assessing androgen receptors in human prostatic carcinoma, especially when only limited amounts of tissue are available. However, contradictory results, with respect to the usefulness of fluorescent steroid conjugates for the detection of steroid hormone receptors in cells or tissues have been published [6–10, 25–34]. Variability in results may have arisen from ill-defined tissue preparations and incubation techniques or impure preparations of steroid conjugates. Tissue sections contain a variety of intact cells, damaged cells, and dead cells, and during incubation decomposition and diffusion of proteins may occur. To circumvent the problems mentioned above, the experiments in this investigation were performed with intact cultured cells or cells that were reproducibly damaged by freezing and thawing. Androgen receptor-positive cells (PC-93 and NHIK-3025) or androgen receptor-negative cells (EB-33) were used. The receptor-positive cell lines appeared to have an intact mechanism for transfer of receptors to the nucleus. The sedimentation values for the receptors in the nuclear extracts (4.6–4.8 S) were in agreement with sedimentation values usually observed for androgen receptors in tissues with low endogenous proteolytic activity [35].

The fluorescent androgenic ligands used in this study contained either BSA or a short hemisuccinate bridge between the steroid and the fluorescent moiety. In the BSA-linked conjugates (T-BSA-FITC) several steroid molecules and fluorescent groups were bound to one albumin molecule, which should theoretically increase the sensitivity of the assay. In the hemisuccinate-linked conjugates (T-HS-FA and DHT-HS-FA) one steroid molecule and one fluoresceinamine molecule were coupled through a short spacer. Coupling of the steroid to fluoresceinamine instead of FITC was chosen because the unconjugated fluoresceinamine does not show fluorescent staining in contrast to unconjugated FITC. The presence of any unconjugated fluoresceinamine in the preparation therefore would not lead to artificial fluorescence.

The relative binding affinities of the fluorescent ligands for the androgen receptor were low. These low binding affinities and, consequently, the high concentration of the compounds required to obtain a reasonable binding to the receptor made

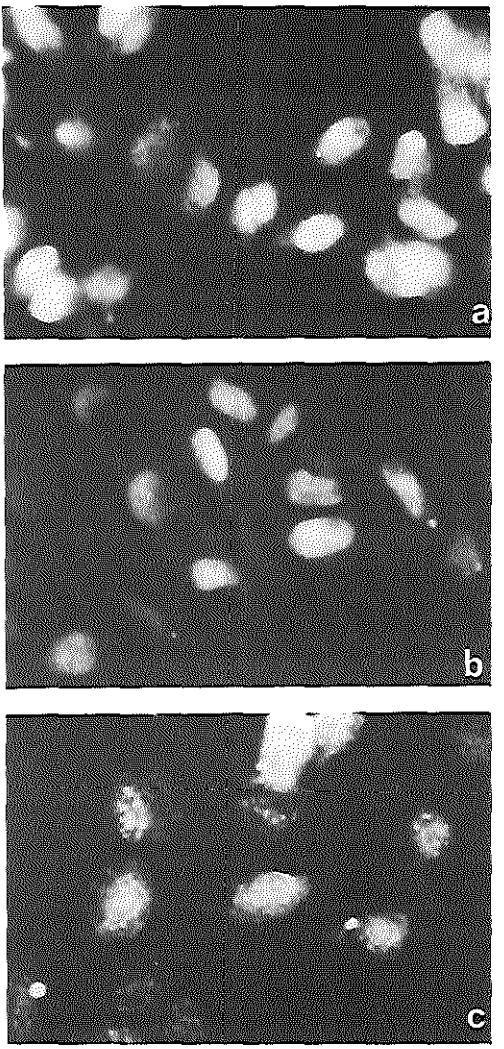

Fig. 4. "Freeze-damaged" PC-93 (a), NHIK-3025 (b), and EB-33 (c) cells stained with 10^{-5} M of T-BSA-FITC for 1 hr, as described in Materials and Methods. Moderate cytoplasmic and intense nuclear staining observed in all three cell lines. ×400.

it likely that nonspecific binding to proteins other than the androgen receptor might occur. Such nonspecific binding could indeed be observed after incubation of the intact cells of the three cell lines with T-HS-FA and DHT-HS-FA. Cytoplasm fluorescence was independent of cell types and occurred in the presence of excess (10^{-7} M) DHT or methyltrienolone. This concentration of DHT or methyltrienolone (both having a high relative binding affinity for the androgen receptor) is enough to saturate all specific high affinity, low capacity binding sites (receptors). When the cells were incubated with 10^{-7} M of the fluorescent ligand (with a low relative binding affinity) and 10^{-7} M of these two potent androgens, *no* reduction in fluorescence is observed when compared to cells incubated with 10^{-7} M of the fluorescent ligand alone, again illustrating the nonspecific character of the fluorescence.

Samples obtained from biopsies of prostate tissue would contain both intact and damaged cells. In this study with model cells we therefore also prepared "freeze-damaged" cells, according to Underwood et al [24], to simulate the freeze/thaw sequence in the preparation of frozen sections. In our study, we used these damaged cells, although the types of damage produced by the freeze/thaw sequence might only reflect part of the damage to cells in tissue sections and biopsy material. Necrotic cells were not studied. The staining of "freeze-damaged" cells with the fluorescent ligands was also studied and again no differences in staining patterns with the different fluorescent ligands were observed. The staining pattern was independent of the affinity of the ligands for the androgen receptor, again illustrating the nonspecific character of the binding. Our results are in agreement with recently published observations. Joyce et al [14] showed that for estrogen-labeled fluorescent conjugates (estrogens coupled through a variety of short spacers), these compounds did not bind to the classical estrogen receptors in thin sections of breast tumor tissue. Moreover, Lammel et al [34] reported that for dihydrotestosterone-FITC conjugates with a variety of short spacers it was not possible to demonstrate androgen receptors in tissue slices obtained from human prostatic carcinomas and human benign prostatic hyperplasia. The absence of specific binding and the consequent disagreement with the biochemical data strongly indicate that the fluorescent conjugates bind predominantly to nonspecific, low affinity binding sites rather than to receptors.

Panko et al [36] reported the presence of a second, specific estrogen binding site in human breast cancer. They observed that the concentration of these type II estrogen binding sites was correlated to the concentration of cytoplasmic receptors. However, the average concentration of these type II binding sites is only four times higher than the concentration of true receptors and therefore hardly visible with normal fluorescence microscopy [27]. In addition, the type II binders were present in nearly all tumors (92% of the 25 investigated tumors contained significant amounts of these type II sites, including six which lacked estrogen receptors).

For the three cell lines used in this study, it appears also that the concentration of these putative low affinity binding sites, estimated with fluorescent ligands, shows no correlation with the concentration of true androgen receptors and therefore cannot be used as a basis for discrimination between androgen receptor-positive and -negative cells. We therefore do not support the claim made by Pertschuk et al [33] that a good correlation would exist between the presence of histochemically characterized androgen binding and the biochemically characterized androgen receptors. Because fluorescent labeled steroids obviously bind to many different cell constituents other than the receptor, a serious overestimation of receptor-positive cells could be made on the

basis of these staining procedures. The results of Pertschuk et al [33] support this view: five of the ten biochemically characterized androgen receptor-negative prostate carcinomas were positive in the histochemical assay.

In conclusion, our results with three different cell lines and several fluorescent labeled androgens do *not* support the view that these fluorescent steroids could be used for the identification of androgen receptor-positive cells in a mixed population of androgen receptor-positive and -negative cells.

ACKNOWLEDGMENTS

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APPENDIX PAPER 3

Histochemical Journal 17, 1185–1196 (1985)

Rapid and sensitive detection of oestrogen receptors in cells and tissue sections by autoradiography with ^{125}I-oestradiol

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Summary

The presence of receptors for steroid hormones in individual cells and tissue sections was assessed within 4–24 h using dry mount autoradiography with radio-iodinated oestradiol. Low affinity and nonspecific binding of steroids were significantly reduced by washing the cells or sections with diluted antiserum to oestradiol.

For cells of the MCF-7 cell line variations in grain density were observed, indicating that cells of the MCF-7 cell line are heterogenous with respect to their cellular receptor concentrations of oestrogen receptors. Receptor-negative cells, such as peritoneal macrophages, did not retain oestradiol label.

In tissue sections of rat and calf uterus, predominant labelling was observed on the endometrial gland cells and stroma.

Oestradiol receptor binding in the uterus cytosol for both radio-iodinated and tritiated oestradiol showed the same qualitative characteristics as determined by sucrose gradient sedimentation profiles and a comparable amount of binding sites was found for both labels. The relative binding affinity of ^{125}I-oestradiol compared to [^{3}H]oestradiol is about 70–80%.

The dry mount autoradiographic technique as presented can be used for rapid screening of heterogeneiety in oestrogen receptor distribution in cells and tissue sections, since this technique reveals differences in receptor concentrations on the single cell level.

Introduction

The presence of oestrogen and progesterone receptors in human breast carcinoma tissue is a good indicator for the positive response of the patient to endocrine therapy (McGuire, 1980). However, a relatively large group of patients (about 30–40%) with positive values for the hormone receptors in tumour tissue, as estimated by biochemical procedures, does not respond to endocrine therapy. Biochemical methods for isolation and characterization of steroid-receptor complexes are time consuming, require special

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equipment and a minimum amount of 100 mg wet weight of tissue, and biochemical analysis of total tissue samples does not give information about the specific cells in the (tumour) specimen which actually contain receptors. Histochemical methods for the visualization of steroid hormone receptors in mammary tumours and in prostatic carcinoma could be of additional value, especially when only limited amounts of tissue are available. Such techniques might also give information about the possible cellular heterogeneity of a tumour which would be useful in predicting more accurately the nature of the response of the patient to endocrine therapy.

Different histochemical techniques have been evaluated for identification of the receptor: (1) autoradiography of radioactive steroids bound to the receptor have been used to visualize directly the steroid (Stumpf & Sar, 1975); (2) antibodies, raised against the steroid hormone have been used to recognize indirectly the steroid bound to the receptor (Nenci *et al.*, 1976); (3) steroid hormones conjugated with fluorescent molecules (Nenci *et al.*, 1980; Pertschuk *et al.*, 1980; Lee, 1981) or inherently or latently fluorescent ligands have been used for direct visualization of the receptor bound oestrogen (Martin *et al.*, 1983); (4) most recently, monoclonal antibodies against the protein moiety of (oestrogen) receptors have been applied (Greene & Jensen, 1982; King & Greene, 1984). With possible exceptions of the autoradiography and the monoclonal antibody techniques, severe doubts exist about the specificity of the histochemical techniques for the recognition of high affinity steroid hormone receptors (Chamness *et al.*, 1980, 1982; McCarty *et al.*, 1980; Berns *et al.*, 1984a,b).

Autoradiography has provided information about the cellular distribution of steroid receptors in tissues that could not have been obtained by biochemical procedures (Stumpf & Sar, 1975, 1976). Generally tritiated ligands were employed to visualize steroid-binding components in cell populations at physiological hormone concentrations (10^{-9}–10^{-10} M). Application of this technique to tumour samples obtained from patients is severely limited by the long exposure time (generally weeks up to months) required to obtain satisfactory autoradiographs. Recently, the synthesis of radio-iodinated 16α-iodo-3, 17β-oestradiol (^{125}I-E_2) was reported (Hochberg & Rossner, 1980). This new ligand has binding characteristics for the oestrogen receptor which are similar to the binding of tritium labelled oestradiol (Hochberg & Rossner, 1980; Tercero *et al.*, 1981; Pieslor *et al.*, 1982; Grill *et al.*, 1983). The iodinated ligand has a high specific activity (approximately 2000 Ci/mmol), hence autoradiographic studies of tissues containing receptors could theoretically be accomplished within hours. In the present study we have investigated whether autoradiography with ^{125}I-oestradiol can be used in a rapid histochemical technique for the detection of oestrogen receptors in cells, cell lines and tissue slices.

Materials and methods

Steroids

2,4,6,7-[^{3}H]oestradiol-17β (91.5 Ci/mmol) and [16α-^{125}I]3,17β-oestradiol, (^{125}I-E_2; 2200 Ci/mmol at the time of dispatching) were obtained from New England Nuclear (Boston, USA). Unlabelled steroids were

purchased from Steraloids (Pawling, USA). The radiochemical purity of the labelled [^{3}H]oestradiol-17β as well as ^{125}I-oestradiol was verified by thin-layer chromatography.

Cell culture

The MCF-7 human breast cancer cell line was provided by the Breast Cancer Animal and Human Tumour and Human Cell Culture Bank, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA. The MCF-7 cells were cultured in a medium 'RPMI-Mix' consisting of RPMI-1640 with L-glutamine (Gibco Europe, UK), 1 liter; containing 10 000 i.u. penicillin, 10 000 μg streptomycin (Gist-Brocades, The Netherlands), 2 mg insulin (Collaborative Research; Waltham, USA) and 10% (v/v) foetal calf serum (FCS) (Boehringer Mannheim, West Germany). Cells were grown at 37° C under a humidified atmosphere of 5% CO_2 in air. Culture flasks, 75 cm^3, were supplied by Costar, (Cambridge, USA). The culture medium was changed twice a week.

Peritoneal macrophages were obtained from male Wistar rats. The peritoneal cavity was rinsed with 25 ml of an 11.6% sucrose solution in phosphate buffered saline (PBS, Gibco Europe, UK). Macrophages were collected from the peritoneal exudate and incubated in RPMI-mix with 1% (v/v) foetal calf serum.

Receptor labelling in cells and tissues, processing for autoradiography

MCF-7 cells and/or macrophages on slides were cultured in RPMI-mix with 10% (v/v) charcoal treated foetal calf serum one day prior to incubation of the cells with labelled steroids. During the labelling with steroids the cells were incubated in RPMI-1640 without serum, for 1 h at 37° C either with ^{125}I-E_2 (1–5 nM) alone or with ^{125}I-E_2 and a 100 fold molar excess unlabelled E_2.

Wistar rats were ovariectomized and uteri were isolated two days later. Rat uteri were trimmed of fat and stored at −80° C. One uterus was mounted on a stud with Rheomacrodex and 4–8 μm sections were cut and mounted on siliconized slides. Calf uteri (obtained from the local slaughterhouse) were trimmed of fat and connective tissues and stored at −80° C. Slices of 0.5 cm were mounted on a stud with Rheomacrodex and 4–8 μm sections were cut and mounted on siliconized slides. The slides, with rat or calf uterus sections, were covered and incubated for 1 h at 20° C in a humidified atmosphere with a few drops 3 nM ^{125}I-E_2 in RPMI-1640 or with 3 nM ^{125}I-E_2 in the presence of a 100-fold molar excess E_2.

The cells or sections were washed twice with PBS, twice with PBS containing 1% bovine serum albumin (BSA) and twice with PBS containing an antibody raised against oestradiol (dilution 1:1000), followed by one wash with PBS containing 10^{-7} M E_2 to exchange low affinity (type II) binders, and once with 0.15 M ammonium acetate (3 min) to exchange ions which cannot be evaporated. The whole washing procedure was performed at 4° C. Every washing step took 10 min, except for the ammonium acetate step. Subsequently, the slides were dipped in liquid Freon and stored in nitrogen and finally freeze dried for at least 24 h. Each slide, cells or tissue down, is placed on another slide previously covered with nuclear emulsion; Ilford K2 (Ilford, UK) (1:1, diluted with aqua bidest). This procedure is essentially the dry-mount autoradiography technique according to Stumpf & Sar, 1975, with the exception of the use of *in vitro* incubated cells or tissues continuously attached to the slides. By pressure between forefinger and thumb, cells or tissue was transferred from the glass slide to the emulsion. The emulsion with adhered cells or tissue was kept at 4° C. After exposure (at 4° C) for 3–24 h (as indicated in the legends of the micrographs), the autoradiographs were developed with Kodak D-19 developer (Kodak-Pathe, France) for 5 min, rinsed with water for 1 min and fixed for 10 min. Cells or sections were counterstained with Haematoxylin alone or with Haematoxylin and azofloxin for 5 s. Excess of dye was removed from the emulsion by washing, the sections were mounted and examined under a microscope and the photomicrographs were recorded on Agfapan 25 films (Agfa-Gevaert, Leverkussen, Germany).

Biochemical receptor analysis

The oestrogen receptor content in MCF-7 cells and peritoneal macrophages, isolated from rat peritoneal

cavity with 11.6% sucrose in PBS, was estimated by sucrose gradient centrifugation as described previously (Berns *et al.*, 1984b).

For the preparation of calf uterus cytosol, calf uteri, stored frozen at −80° C were cut with scissors and homogenized at 0° C with an ultrathurrax in four volumes 40 mM Tris, 1.5 mM EDTA, 1.5 mM dithiothreitol and 10% (v/v) glycerol. The homogenate was centrifuged at 10 000*g* for 10 min followed by removal of the lipid layer. The cytosol was prepared by centrifugation of the supernatant for 60 min at 100 000*g*.

Estimation of the oestrogen receptor content using sucrose gradient centrifugation, protamine sulphate precipitation assay and protein determination was performed as described previously (Berns *et al.*, 1984b).

Relative binding affinity

The binding affinity of ^{125}I-E_2 for the oestrogen receptor obtained from uterine cytosol from a two days ovariectomized rat, was estimated as described previously (Berns *et al.*, 1984b). Dextran-coated charcoal was used for the separation of free and bound steroid.

Results

In this study we used cells from a breast tumour line, MCF-7, and peritoneal macrophages. These cells can be discriminated not only on the basis of their biochemically characterized oestrogen receptor content, but also on microscopical examination, revealing a remarkable difference in size. Rat and calf uterus were used as a second test system for which the oestrogen receptor content was known. First we have compared the labelling of the receptor with [^{3}H] and ^{125}I-oestradiol with biochemical procedures and subsequently we have performed autoradiography of the labelled ligand receptor complex *in situ*.

Biochemical characterization of ^{125}I-E_2 binding

The binding of ^{125}I-E_2 and [^{3}H]E_2 to the oestrogen receptor were compared in both intact MCF-7 cells and calf and rat uterus cytosol, because the iodine on the 16-position of oestradiol might have influenced the binding of oestradiol to the receptor. Cells or cytosol were incubated with non-saturating concentrations (3 nM) of ^{125}I-E_2. Incubations with higher concentrations were not carried out because of the high price of ^{125}I-E_2. After sucrose gradient centrifugation of the nuclear extracts we observed one major peak at 4 S for both [^{3}H]E_2 and ^{125}I-E_2 (Fig. 1), indicating that the ^{125}I-E_2 and [^{3}H]E_2 labelled steroid-receptor complexes were similar in MCF-7 cells and uterus cytosol. The amount of oestrogen bound to receptor measured with 3 nM ^{125}I-E_2 was also comparable to the amount measured with 3 nM [^{3}H]E_2. The amount of binding sites measured with 3 nM E_2 is approximately half the amount of binding sites found with a saturating (10 nM) concentration of [^{3}H]E_2 (Table 1).

Peritoneal macrophages were used as control cells for the autoradiographic studies. With biochemical procedures (sucrose gradient centrifugation of nuclear extracts) no oestrogen receptors could be detected in these cells.

The relative binding affinity of labelled oestradiol was estimated by incubation of uterine cytosol with 2 nM ^{125}I-E_2 or [^{3}H]E_2 and increasing amounts of unlabelled E_2.

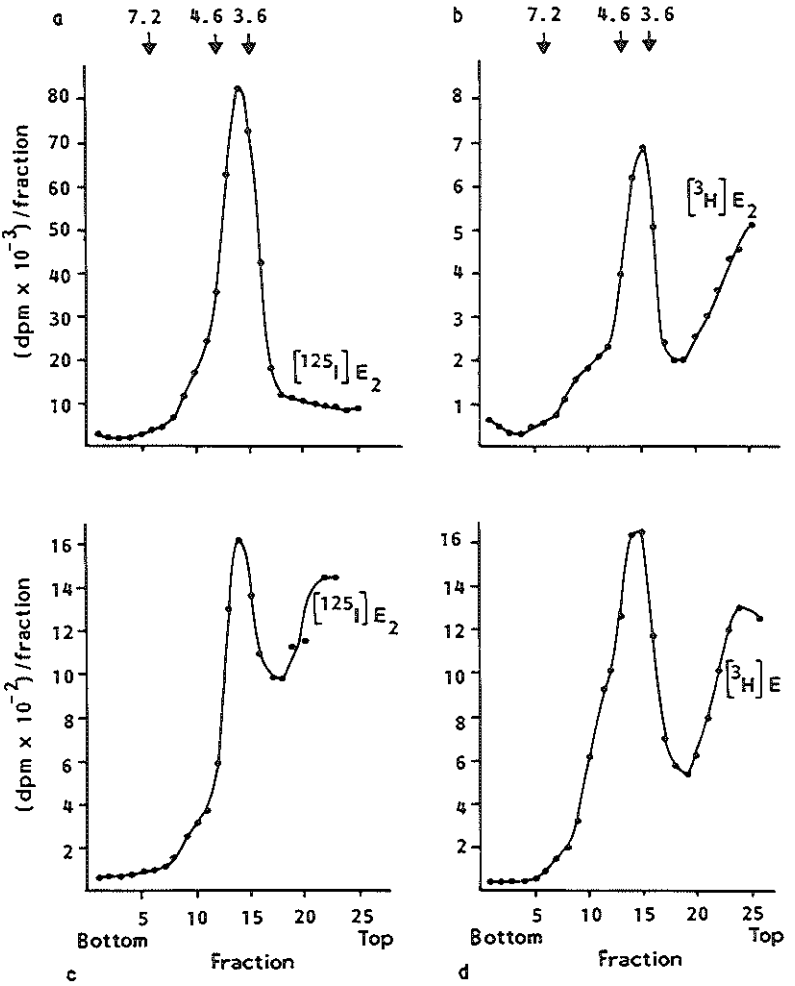

Fig. 1. Sucrose gradient sedimentation profiles of cytosol from calf uterus incubated for 2 h at 0° C with 3 nM ^{125}I-E_2 (a) and 10 nM [^{3}H]E_2 (b) or nuclear extracts from MCF-7 cells incubated for 1 h at 37° C with 3 nM ^{125}I-E_2 (c) and 10 nM [^{3}H]E_2 (d). The concentration of the cells in (d) was five times higher as in (c). The 90–200 μl cytosol or nuclear extract was layered on the sucrose gradients (5–20%), containing 0.4 M KCl. Gamma globulin (γ-G; 7.2 S), bovine serum albumin (BSA; 4.6 S) and ovalbumin (OV; 3.6 S) were used as sedimentation markers.

Table 1. Oestrogen receptor content estimated in calf and rat uterus cytosol after incubation with either 3 nM ^{125}I-E_2, 3 nM [^{3}H]E_2 or 10 nM [^{3}H]E_2.

	Oestrogen receptor (fmol/mg *protein*)		
	^{125}I-E_2 (3 nM)	[^{3}H]E_2 (3 nM)	[^{3}H]E_2 (10 nM)
Calf uterus cytosol	265	256	686
Rat uterus cytosol	245	351	595

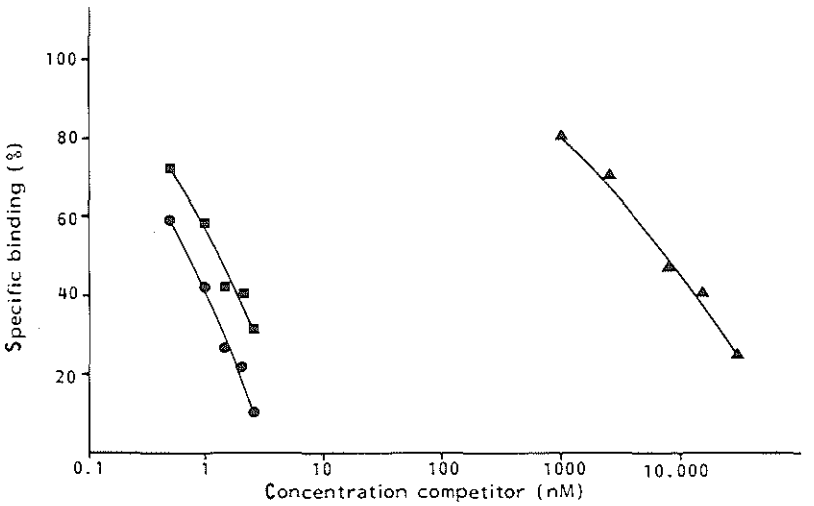

Fig. 2. Binding affinity of various ligands for the oestrogen receptor from uterine tissue. Cytosol was prepared from uteri of mature ovariectomized rats, as described in the Methods section, and incubated with labelled oestradiol and increasing amounts of unlabelled ligands. Specific binding of labelled oestradiol in the absence of the unlabelled competitors was set at 100%. ●, 1.5 nM ^{125}I-oestradiol with increasing amounts of unlabelled oestradiol; ■, 2.2 nM [^{3}H]oestradiol with increasing amounts of unlabelled oestradiol; ▲, 10 nM [^{3}H]oestradiol with increasing amounts of unlabelled oestradiol-17-hemisuccinate-fluoresceinamine.

Percentage binding was measured by the charcoal technique. Relative binding affinities of the ligands for the oestrogen receptor in rat uterus are presented in Fig. 2. The calculated binding affinity of ^{125}I-E_2 was about 70–80% compared to unlabelled oestradiol.

Autoradiography

Initial experiments using MCF-7 cells labelled with ^{125}I-E_2 and subsequently processed for autoradiography revealed grains over the cells and also grains which were not associated with cells. This nonspecific binding could be reduced by washing with an antibody raised against oestradiol.

Steroid hormones can bind to specific receptors and nonspecifically to other proteins. The high affinity, specific sites are present in limited amounts. We have tested the reliability of the receptor binding assay by incubating the cells or tissue slices in the presence of a large excess non-radioactive steroid, followed by extensive washing. Under this condition very few specific sites are labelled (because much less radioactive steroids bind to the receptors) and after washing the autoradiographs reveal only a limited amount of grains above the cells.

To test for the presence of low affinity binding components in addition to oestrogen receptors we have incubated the MCF-7 cells with ^{125}I-E_2 and a 100-fold molar excess of unlabelled oestradiol and again washed with an antibody against oestradiol. In these autoradiographs we hardly observed any grains above the cells (Fig. 3a). Both MCF-7 cells and peritoneal macrophages are shown in Fig. 3b. Macrophages are consistently smaller in size compared to the MCF-7 cells. In this preparation we can also observe that the morphology of the MCF-7 cells is reasonably well preserved during the autoradiographic procedure (e.g. note the preservation of the nucleoli). In the sections of rat uterus tissue incubated with ^{125}I-E_2 plus a 100-fold molar excess of oestradiol we observed an equally good preservation of the endometrial structure. Hardly any grains above the endometrial stroma and gland cells were observed.

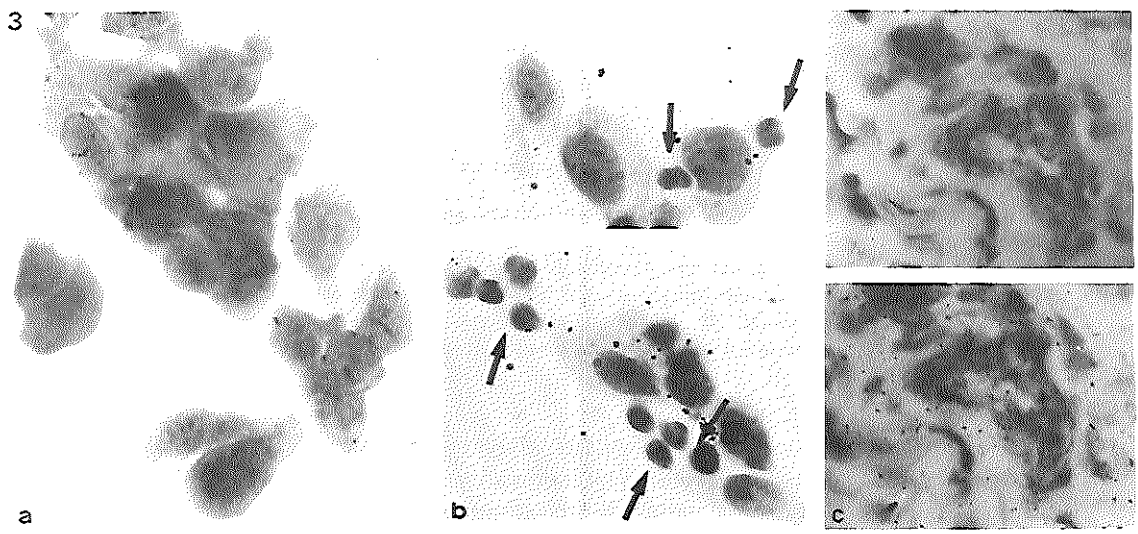

Fig. 3. Nonspecific labelling. Dry-mount autoradiographs of cells incubated with 5 nM ^{125}I-E_2 and 500 nM E_2, for 1 h at 37° C, and sections incubated with 3 nM ^{125}I-E_2 and 300 nM E_2 for 1 h at 20° C. The excess non-radioactive steroid prevents binding of ^{125}I-E_2 to receptor molecules. (a) MCF-7 cells, islet. (b) MCF-7 cells and macrophages (small cells, arrows). (c) Section of rat uterus (endometrial and stroma gland), with focus on grains. (d) Same section as in (c) with focus on tissue. The exposure time was 18 h and section thickness 8 μm. × 1035.

The autoradiographs of cells and tissues with ^{125}I-E_2 labelled receptors are shown in Figs. 4 and 5. After incubation of the MCF-7 cells with ^{125}I-E_2 and subsequent autoradiography the silver grains were only localized over the cells (Fig. 4a). This figure shows an example of an islet of MCF-7 cells with intense grain density above the cells. Heterogeneiety of labelling is shown in Fig. 4b,c; not all cells show the same grain density and some MCF-7 cells did not accumulate silver grains. In addition Fig. 4b shows some macrophages, which do not contain oestrogen receptors and do not accumulate silver grains over the cells.

In sections of rat as well as of calf uterus incubated with ^{125}I-E_2 (3 nM) the silver grains were localized over the endometrial gland cells and stroma (see Fig. 5).

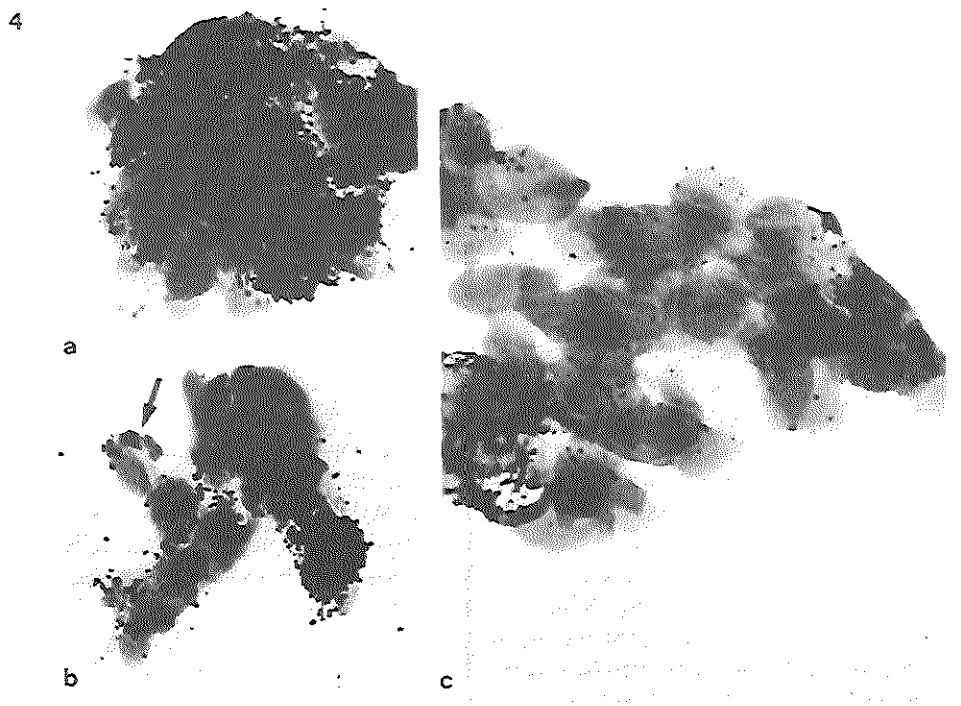

Fig. 4. Specific labelling of cells. Dry-mount autoradiographs of cells incubated with 5 nM ^{125}I-E_2 for 1 h at 37° C. (a) MCF-7 cells, islet, intense labelling. (b) MCF-7 cells, and macrophages (small cells, arrow). (c) MCF-7 cells, note the heterogeneity of labelling. The exposure time varied between 19–24 h. (a) and (b) × 1125; (c) × 1250.

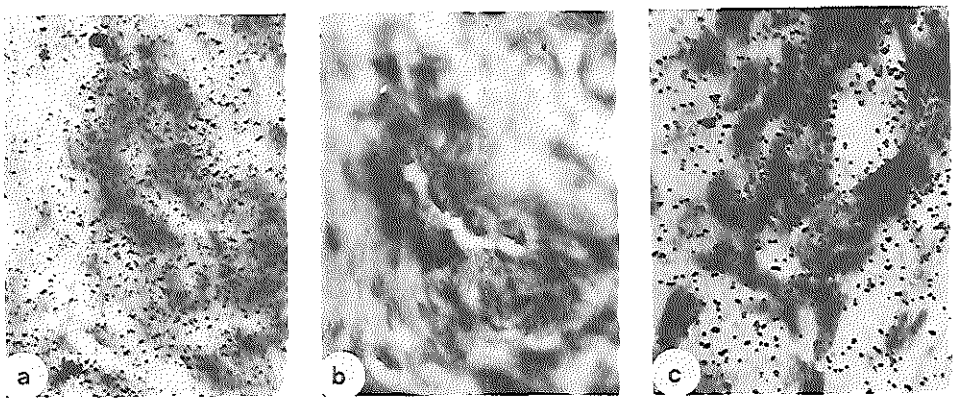

Fig. 5. Specific labelling of tissue sections. Dry-mount autoradiographs of sections incubated with 3 nM ^{125}I-E_2 for 1 h at 20° C. (a) Rat uterus, endometrial stroma and gland with focus on grains. Note labelling of glandular and stromal cells. (b) The same section, as in (a) with focus on tissue. (c) Calf uterus, endometrial stroma and gland, note labelling of glandular and stromal cells. The exposure time varied between 19–24 h, section thickness 8 μm. × 1125.

Discussion

We have evaluated dry mount autoradiography using ^{125}I-labelled oestradiol for autoradiographic detection of oestrogen receptors in specific cells and tissue sections with biochemically estimated receptor concentrations. MCF-7 cells derived from a breast tumour, known to be oestrogen receptor positive and peritoneal macrophages, oestrogen receptor negative, were used for these studies. Introduction of the iodine atom at the 16α-position of oestradiol might influence the binding of the ligand to the receptor, and we have compared the binding of ^{125}I-E_2 and [^{3}H]E_2 to intact mammary tumour cells. From the results we have concluded that the ^{125}I-label binds to the same specific nuclear 4 S oestrogen receptor as does the ^{3}H-label (see Fig. 1).

Our results also showed that the affinity of ^{125}I-E_2 and [^{3}H]E_2 for the receptor was comparable. We conclude therefore that ^{125}I-E_2 and [^{3}H]E_2 behave similarly in both intact cells as well as in cytosol preparations. These results are in agreement with the observation of Hochberg, who also noticed a specific uptake of unmetabolized ^{125}I-oestradiol by the uterus of castrated female rats (Hochberg, 1979; Hochberg & Rossner, 1980).

Autoradiographic techniques using freeze dried sections, dry mounted on photographic emulsions exclude sources of diffusion artefacts like liquid fixation, embedding, wet tissue and wet emulsion mounting (Stumpf, 1968). In our modification of this

technique we have introduced a special washing procedure which greatly reduced the nonspecific steroid binding. The use of an antibody raised against oestradiol and the chase with a hundred fold molar excess of radioinert oestradiol contributed especially to the detection of receptors only. Above oestrogen receptor negative cells (e.g. peritoneal macrophages), no silver grains were observed.

In our studies, the dry mount autoradiographs of MCF-7 cells incubated with ^{125}I-E_2 revealed silver grains over most of the cells. Studies with rat or calf uterus sections, confirmed the results with MCF-7 cells. After incubation of calf uterus sections with ^{125}I-E_2, the silver grains appeared over the endometrial gland cells and endometrial stroma cells.

Differences in grain density were not only observed between cells with different functions in the tissue sections but also between cells of the MCF-7 cell line. This heterogeneiety probably reflects biological variation, which could be due to transient alterations in receptor levels related to changes in cell cycle (Stumpf, 1968). The difference in grain density was first observed by Stumpf (1968) with [^{3}H]E_2 in rat uteri. Similar observations were made by Buell & Tremblay (1981) in mouse uteri.

Using monoclonal antibodies directed against the human oestrogen receptor, King & Greene (1984) observed a heterogenous pattern of nuclear staining in the MCF-7 cells. This heterogenous nuclear staining was also observed by Press & Greene (1984) with the same antibody (H226Sp) on frozen sections of human uteri. Both the autoradiographic techniques, with [^{3}H]E_2 or ^{125}I-E_2, as well as the immunocytochemical method, using antibodies directed against the human oestrogen receptor, reveal a similar receptor pattern and support the conclusion that the observed heterogeneiety in labelling reflects a real heterogeneiety in receptor distribution in different cells.

The present dry mount autoradiography with ^{125}I-oestradiol provides a rapid and sensitive histochemical technique for detection of the steroid binding site of oestrogen receptors, in intact cells as well as in tissue sections. Depending on the specific activity of the label the required exposure time may vary from 4–24 h, which makes it possible to use this method for a relatively fast screening. Application of this technique on samples of mammary tumours may be attractive for the detection of receptor concentrations and its variation on the cellular level in conjunction with a morphological evaluation.

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APPENDIX PAPER 4

COMBINED TECHNIQUES OF AUTORADIOGRAPHY USING ^{125}I-OESTRADIOL AND IMMUNOCYTOCHEMICAL ASSAY, USING MONOCLONAL ANTIBODIES TO THE HUMAN OESTROGEN RECEPTOR, FOR DETECTION OF THE OESTROGEN RECEPTOR

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Running title: Detection of oestrogen receptors with combined autoradiography and immunocytochemistry

Keywords: oestrogen receptor, autoradiography, monoclonal antibodies, MCF-7 cells

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Abbreviations used: ER, oestrogen receptor; ER-ICA, oestrogen receptor immunocytochemical assay; E_2, oestradiol; I, iodine; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute (medium).

SUMMARY

The presence of oestrogen receptors in MCF-7 cells is demonstrated with autoradiography, using (^{125}I)-oestradiol, an immunocytochemical assay, using a monoclonal antibody (H222Spy) directed against the oestrogen receptor from MCF-7 cells and the combined autoradiography-immunocytochemistry. The MCF-7 cells showed variations in grain density and also variations in immunostaining, indicating that the cells of the MCF-7 cell line are heterogeneous with respect to their cellular receptor concentrations. The immunostaining and grains were confined to the nucleus. A control antibody did not reveal immunostaining and incubation of the cells with a 100-fold molar excess of unlabelled oestradiol did not reveal silver grains above the cells, indicating that only oestrogen receptors are demonstrated by both techniques.

The combined autoradiography and immunocytochemistry showed that 68±18% of the cells are immunostained and reveal grains above the same cells, whereas 30±16% of the cells were not stained and did not reveal silver grains above them. There was a good agreement (98%) between the presence of grains and specific immunostaining. In 2% no silver grains were observed above the nuclei of specific immunostained cells. This technique might be useful for the study of (malignant) cells with receptor systems with defects located in the region of the steroid binding site.

INTRODUCTION

Oestrogens excert their biological effects through an interaction with intracellular receptors. The steroid-receptor complexes thus formed can interact with regulatory sites on the genome. Target tissues contain a variety of cell-types that may exhibit unique responses to the same hormone. Biochemical assays generally do not discriminate among different tissue components, and several histochemical techniques have been developed to identify receptors in different cell types. Dry mount autoradiography with tritium labelled hormones has been shown to be a reliable method for the detection of steroid binding proteins in cells (Stumpf et al, 1968). Due to the long exposure times involved, this technique has not been applied routinely for the detection of steroid hormone receptors in tumour tissues. Several techniques aiming at a fast

detection of oestrogen receptors using either antibodies against conjugated oestrogens or the fluorescent conjugated oestrogen assay have been developed to fill the need for a fast histochemical detection of oestrogen receptors (Pertschuk et al, 1979; Lee, 1979). Serious questions have arisen concerning the ability of such assays to detect receptors (e.g. Chamness et al, 1980). Indeed, with such techniques steroid receptors can not be detected (Berns et al, 1984a,b; Lämmel et al, 1984). Recently fast histochemical assays became available, which may rapidly provide data on cellular localization of the steroid hormone receptors. Firstly, monoclonal antibodies raised against the oestrogen receptor became available for histochemical studies on oestrogen receptor localization (King and Greene, 1984). Second, a dry mount autoradiographic technique using (^{125}I)-oestradiol, with a high specific activity, has been developed in our laboratory for the rapid visualization of the oestrogen receptor (Berns et al, 1985). These two techniques depend on different principles for visualization of the receptor. The autoradiographic technique may reveal the steroid binding properties of the receptor proteins, whereas the commercially available antibody (H222spy) reveals the steroid binding domain (Greene et al, 1984), but not the steroid binding site, of the oestrogen receptor molecule. It is conceivable that the immunocytochemical assay also reveals immuno-reactive receptor molecules without steroid binding capacity. On the other hand the autoradiographic technique detects steroid molecules not bound to receptors.

We have observed changes of oestrogen receptor levels in Leydig cells during culture and suggested that during culture of the cells either the binding properties of the oestrogen receptor might change or that the binding site is inactivated or that the receptor molecule is absent (Berns et al, 1984). Since the immunocytochemical assay and the autoradiography reveal one of the above mentioned suggestions, it is possible to investigate these suggestions seperately.

In the present study we show that individual cells in a non-synchronized human mammary tumour cell culture (MCF-7 cell line) show differences with respect to their oestrogen receptor content (varying from strongly receptor positive to receptor negative cells). In addition we demonstrate that there is a good agreement between the results of the autoradiographic and the immunocytochemical assay.

MATERIALS AND METHODS

Steroids.

(16α -^{125}I)3,17β -oestradiol (2200 Ci/mmol at the time of dispatching) was obtained from New England Nuclear (Boston, Massachusetts). Unlabelled oestradiol was purchased from Steraloids (Pawling, New York).

Cell culture.

The MCF-7 human breast cancer cell line was provided by the Breast Cancer Animal and Human Tumour Cell Culture Bank, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. The MCF-7 cells were cultured in a medium RPMI-mix consisting of RPMI-1640 with L-glutamine (Gibco Europe, United Kingdom); 1 liter, containing 10.000 IU penicillin, 10 mg streptomycin (Gist-Brocades, The Netherlands), 2 mg insulin (Collaborative Research: Inc. Waltham Mass.) and 10% (v/v) foetal calf serum (Gibco). Cells were grown at 37°C under a humidified atmosphere of 5% CO_2 in air. Culture flasks, 75 cm^2, were supplied by Costar (Cambridge, Mass.). The culture medium was changed twice a week.

Receptor labelling in cells.

MCF-7 cells cultured on slides were grown in RPMI-mix with 10% (v/v) foetal calf serum treated twice with dextran coated charcoal to remove endogenous steroids one day prior to incubation of the cells with labelled steroid. During the labelling with steroids the cells were incubated in RPMI-1640 without serum, for one hour at 37°C either with (^{125}I)-oestradiol (1-5 nM) or with (^{125}I)-oestradiol and a 100-fold molar excess unlabelled oestradiol.

Processing for autoradiography.

Autoradiography was performed as described by Berns et al, (1985). Shortly: the cells were washed twice with phosphate buffered saline, twice with phosphate buffered saline containing 1% bovine serum albumin and twice with phosphate buffered saline containing an antibody against oestradiol

(dilution 1:1000), followed by washing once with phosphate buffered saline containing 10^{-7} M oestradiol, to exchange low affinity and high dissociation (type II) binder, and once with 0.15 M ammonium acetate to exchange ions which cannot be evaporated. The whole washing procedure was performed at 4°C. Most washing steps took on average 10 minutes, except for the ammonium acetate step which was performed in 3 minutes. Subsequently, the slides were dipped in liquid Freon, kept in N_2 and subsequently dried for at least 24 hours. Dry mount autoradiography was performed according to Stumpf et al, (1975). Cells were dry mounted onto glass slides previously coated with Illford K2 emulsion (Illford, Essex, United Kingdom), (1:1, diluted with double distilled water).

Combined autoradiography and immunocytochemistry.

At the end of the exposure time for autoradiography (24-48 hours as indicated in the figures, at 4°C) the cells were fixed in 3.5% formaldehyde in phosphate buffered saline at 4°C for 1 minute, prior to photographic development. The emulsion was developed in Kodak D19 developer diluted with phosphate buffered saline (1:1) for only 1 minute at room temperature, washed with phosphate buffered saline and fixed, with rapid fixer, for only 1-2 minutes. Cells were washed twice (for 5 minutes) with phosphate buffered saline and subsequently processed for immunocytochemical assay using a monoclonal antibody H222Spy against the human oestrogen receptor as recommended by the manufacturer, as described by Blankenstein et al (1986). The ER-ICA-kit was a gift of Abbott Diagnostics, Chicago). In some cases no counterstain was used and in other cases cells were counterstained with Harris haematoxylin (1:10 diluted) for only ten seconds (as indicated in the results). After embedding the cells were examined under a microscope and the photomicrographs were recorded on Kodak Ectachrome 160 films (Kodak, Rochester, N.Y.).

RESULTS

Because the introduction of the iodine atom on the 16α-position of estradiol might influence the transport of the labelled estradiol through the cell membrane or the binding of the labelled estradiol to the receptor, we have compared the sedimentation profiles binding of (^{125}I)-E_2

and (^{3}H)-E_2 to the oestrogen receptor in intact MCF-7 cells. After sucrose gradient centrifugation of the nuclear extracts we observed one major peak at 4S for both the tritiated and the iodinated oestradiol , indicating that the (^{125}I)-E_2 and (^{3}H)-E_2 labelled steroid-receptor complexes were similar in MCF-7 cells (Berns et al, 1985).

Immunocytochemistry.

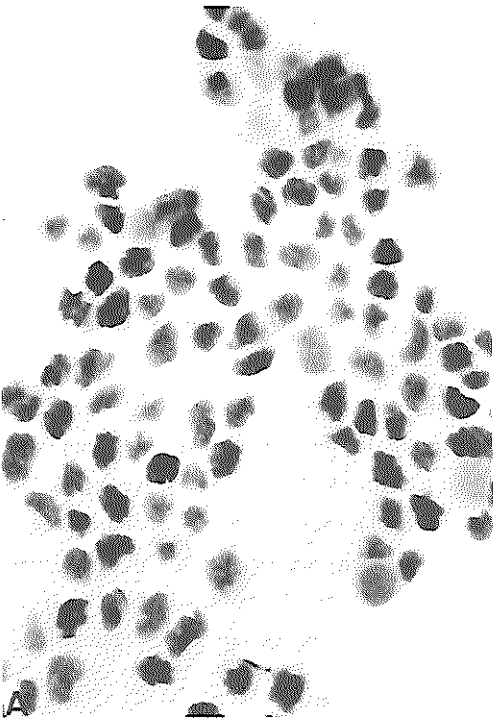
Cultured MCF-7 cells were fixed and processed for immunocytochemistry. An Abbott ER-ICA monoclonal control slide was also included in this test. After incubation with the monoclonal anti-oestrogen receptor antibody and peroxidase staining, we observed specific staining for oestrogen receptors in the cells in the control slide and in the MCF-7 cells. This specific staining was heterogeneous and confined to the nuclei (see figures 1A and 1C). Some MCF-7 cell nuclei did not stain, whereas other cell nuclei were weakly stained. Parallel incubations of the control slide and MCF-7 cells with a control antibody did not reveal nuclear staining (see figures 1B and 1D).

Autoradiography.

Cultured MCF-7 cells were incubated with 3 nM (^{125}I)-oestradiol and subsequently processed for autoradiography. We observed MCF-7 cells with a heterogeneous labelling of the cells. Some cells show an intense grain density above them whereas others did not show no grains above the cells. In a parallel experiment we incubated the MCF-7 cells with 3 nM (^{125}I)-oestradiol with a 100-fold molar excess of unlabelled oestradiol. Hardly any grains were observed above these cells, indicating that the non-specific labelling is low (Berns et al, 1985).

Combined autoradiography-immunocytochemistry.

MCF-7 cells were incubated with 3 nM (^{125}I)-oestradiol and subsequently processed for autoradiography and immunocytochemistry with a monoclonal antibody against the oestrogen receptor. Figure 1E shows an example of an islet of MCF-7 cells. The oestrogen receptors were localized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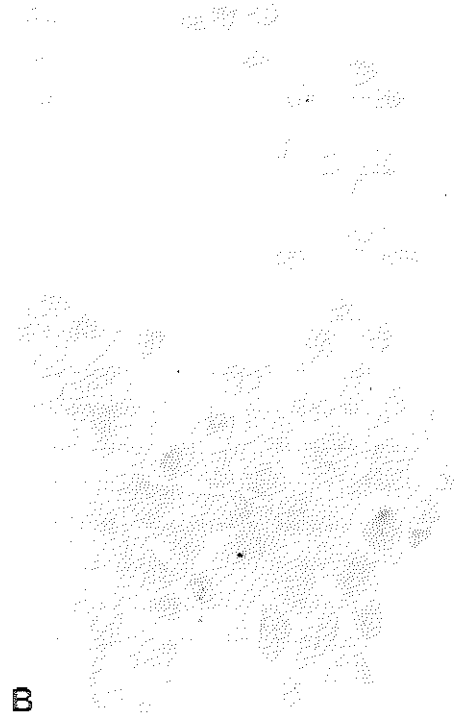
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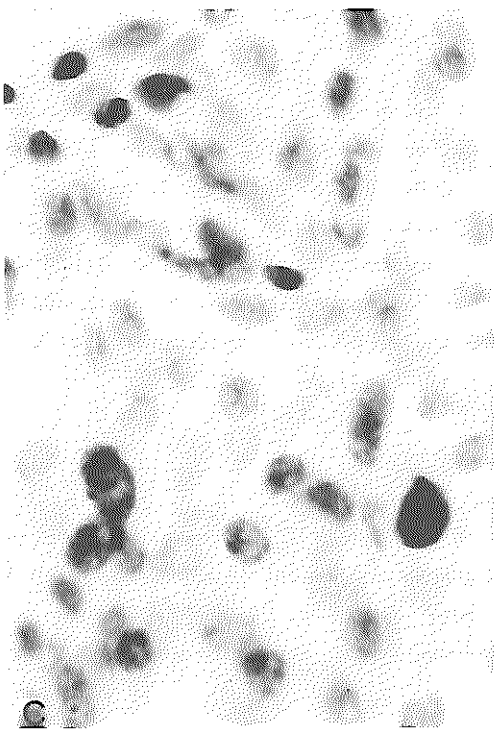
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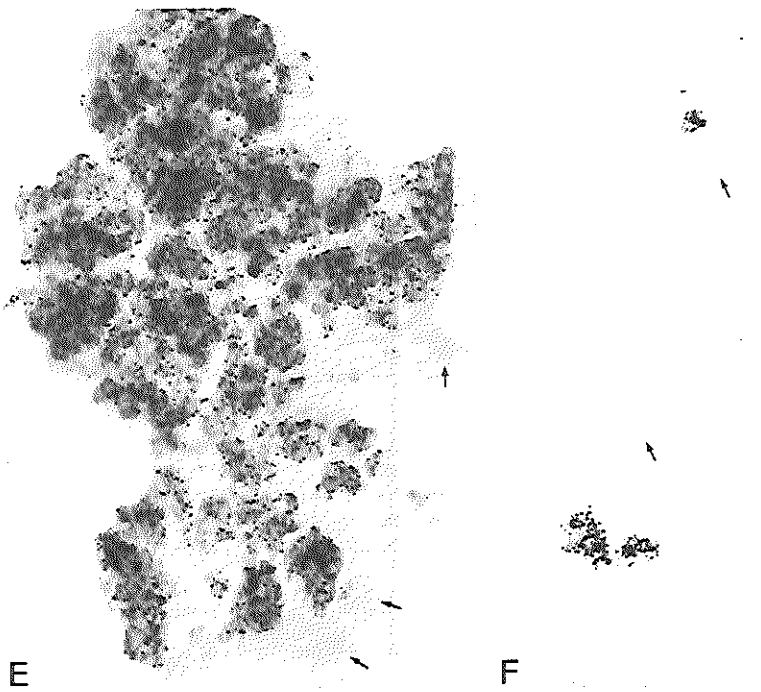

Figure 1.
A) Abbott ER-ICA monoclonal control slide stained using primary antibody.
B) Abbott ER-ICA monoclonal control slide stained using control antibody.
C) MCF-7 cells stained using primary antibody.
D) MCF-7 cells stained using control antibody.
E) Immuno-autoradiogram of an islet of MCF-7 cells. Cells were incubated with 3 nM (^{125}I) oestradiol. Dry mount autoradiography was performed with subsequent immunostaining with the primary monoclonal antibody. The exposure time was 24 hours.
F) As in E, some single cells. Without haematoxylin counterstaining.
Arrow indicates the cells without grains and peroxidase stain.
Magnification 630x.

in most of the cells using the monoclonal antibody and sensitive immunoperoxidase techniques. Above the same immunoreactive cells the silver grains formed after exposure of the emulsion to radioactive steroid were also visible. Incubation of the MCF-7 cells with 3 nM (^{125}I)-E_2 in the presence of a 100-fold excess of oestradiol and processed for combined autoradiography-immunocytochemistry showed immunostaining with hardly any grains above the cells (not shown). This demonstrates that the formation of grains can be suppressed by excess steroid and indicates that the grains formed after incubation with 3 nM (^{125}I)-E_2 only reflect binding sites with a high affinity and a low capacity, both

Table 1. Correlation between autoradiographic and immunocytochemical data.

		ERICA	
		pos	neg
AUTORAD	pos	68 ± 18	0
	neg	2 ± 2	30 ± 16

In 3 MCF-7 cell preparations at least 500 cells were counted with respect to immunostaining and the presence of silver grains resulting from the bound irradiation by (^{125}I)-labelled oestradiol. Cells with 2 or more grains above the nucleus were considered positive. (Background grains were substracted).

characteristics of the oestrogen receptor. The combined autoradiography and immunocytochemical staining showed a concentration of silver grains above, and dark brown diaminobenzidine reaction product on the nuclei of the majority of the MCF-7 cells, whereas other MCF-7 cells remained unlabelled and were not immunostained.

There is a good agreement between the presence or absence of silver grains and specific immunostaining of the cells (as shown in table 1). In 98% of the cells there was agreement between the results obtained with immunocytochemistry and autoradiography, either demonstrating the presence or absence of receptors. In 2% (±2) of the cells no silver grains were observed above specific immunostained nuclei. In one preparation the number of grains was counted per immunoreactive nucleus. The result is shown in figure 2. In this preparation a large group of cells (60%) showed 1-10 silver grains per nucleus; the remainder of the cells showed a considerable higher amount of grains, probably due to a large individual variation in receptor content of the cells.

DISCUSSION

In non-synchronized MCF-7 cells with biochemically estimated receptor concentration, we have compared dry mount autoradiography using (^{125}I-iodi-

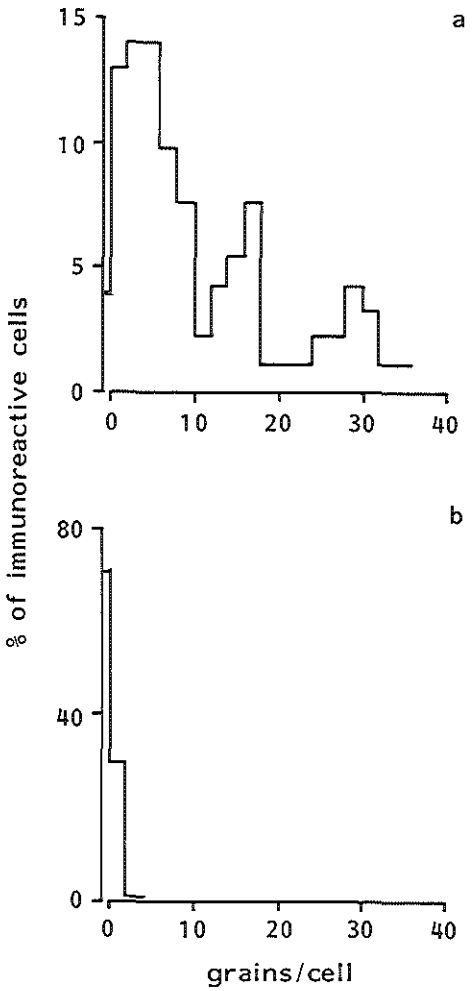

Figure 2. Number of silver grains per immunoreactive nucleus in radioautograms of MCF-7 cells labelled with (^{125}I)-oestradiol. MCF-7 cells were incubated for 1 hour with 3 nM (^{125}I)-oestradiol (2a) or 3 nM (^{125}I)-oestradiol with a 100-fold molar excess of unlabelled oestradiol (2b) and processed for autoradiography and immunocytochemistry as described in the Materials and Methods section. Exposure time for autoradiography was 24 hours. The number of nuclei with a corresponding number of grains is presented in the histograms as percentage of total nuclei.

nated oestradiol for autoradiographic detection of oestrogen receptors and an immunocytochemical assay using monoclonal antibodies raised against the human oestrogen receptor.

Autoradiographic techniques using freeze-dried cells, dry mounted on photographic emulsions exclude possible sources of diffusion artifacts like liquid fixation, embedding, wet tissue and wet emulsion mounting (Stumpf, 1968). The use of the iodine label with high specific activity considerably shortened the exposure time in the autoradiographic procedure when compared to the original procedure as used by Stumpf (1968). In addition washing of the preparations with an antibody directed against oestradiol decreased the

non-specific binding to less than one grain per cell in our standard procedure (Berns et al, 1985). We have compared the localization of sites for steroid hormone binding with the localization of an antibody raised against a site on the oestrogen receptor close to, but not identical with the steroid binding site (H222spy; Greene et al, 1980, King and Greene, 1984). We observed that the immunoreactive MCF-7 cell nuclei were essentially identical to the nuclei that revealed silver grains, reflecting bound (^{125}I)-oestradiol.

The MCF-7 cell culture was particularly suitable for this study because these cells showed a heterogeneous distribution of the receptor in culture. Approximately 30% of the cells did not contain receptors and also within the group of receptor positive cells a variation in grain count and intensity of immunostaining was observed. A comparable percentage of oestrogen receptor negative cells was reported by Kodana et al, (1985) with a mixture of 2 different monoclonal antibodies (D547 and D75) obtained from Greene. They suggest that the occurence of oestrogen receptors increases with clonal growth and appears to be expressed in the differentiation process. The heterogeneity in receptor content of the MCF-7 cells might reflect biological variations in receptor levels related to changes in the cell cycle, especially in the G_1 and G_2 phase as has been observed by Jakesz et al, (1984), in partially synchronized MCF-7 cells. This has also been observed for glucocorticoid receptors in synchronized HeLa cells during the S-phase of the cell cycle (Cidlowsky and Michaelis, 1977).

The results in this report demonstrate a good correlation between accumulation of (^{125}I)-E_2 and the recognition of the antigenic site on the steroid binding domain, by specific monoclonal antibodies directed against the human oestrogen receptor. Only approximately 2% of the cells with specific immunostaining did not accumulate sufficient labelled steroid for the formation of silver grains, during the exposure time. A possible explanation could be that the level of receptors in these cells was too low to be detected via binding of (^{125}I)-oestradiol and formation of silver grains above these cells, whereas the immunostaining might have been more sensitive. On the other hand O'Malley et al, (see King, 1985) recently demonstrated the existence of immunoreactive molecules without steroid binding capability but immunologically indistinguishable from a progesterone receptor in the same

preparation. Our results do not permit a definite conclusion about the nature of the antigen in the minor fraction of peroxidase positive cells that do not accumulate grains above the nucleus.

Both techniques demonstrate oestrogen receptors, however since the monoclonal antibody technique is much more easier to perform and this technique also reveals a clear morphology we prefer this technique for a more widespread use.

In conclusion the present results demonstrate that with a combination of autoradiography and immunocytochemistry it is possible to study heterogeneity of cells with respect to both the steroid binding and the receptor moiety in one preparation. This technique might especially be useful for study of malignant cells with an abnormal system with defects located in the region of the steroid binding site.

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APPENDIX PAPER 5

ANDROGEN DEPENDENT GROWTH REGULATION OF AND THE RELEASE OF SPECIFIC PROTEIN(S) BY THE ANDROGEN RECEPTOR CONTAINING HUMAN PROSTATE TUMOR CELL LINE LNCaP

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Running title: Androgen effects on the LNCaP cell line

Keywords: Androgen receptors; prostate tumor; cell line; LNCaP; released proteins; growth

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ABSTRACT

Hormone sensitivity, as indicated by the presence of steroid hormone receptors and the effect of androgens and anti-androgens on the release of proteins by cultured cells of the human prostate tumor cell line, LNCaP-FGC, has been studied.

The growth of the LNCaP-FGC cells was stimulated by androgens in a dose-dependent way. Under optimal conditions the synthetic non-metabolizable androgen R1881 (0.1 nM) stimulated cell growth approximately 2.3 times. Increasing doses of R1881 (1-100 nM) partly decreased the stimulation of the cell growth. The anti-androgen cyproterone acetate exerted inhibitory effects on cell growth. The nuclear extract of the LNCaP-FGC cells contained 17,000 ± 2,500 (mean ± S.D.) KCl-extractable, nuclear androgen receptor sites/cell. Estrogen and progesterone receptors were not detectable in the nuclear extracts nor in cytosol, indicating that these receptors are absent.

The release of proteins in the culture medium was studied using incorporation of (^{35}S)-methionine, SDS-gelelectrophoresis and fluorography. Cells grown in media containing charcoal-stripped fetal calf serum released significantly lower amounts of a protein with an apparent molecular mass of 40,000 Dalton. The release of this 40 kD protein could be restored in cells cultured in the presence of DHT (0.1-1 μM) or R1881 (0.1 nM - 100 nM), whereas the addition of estrogens or corticosteroids had no effect. In the presence of anti-androgens, such as cyproterone acetate and RU 23908, inhibitory effects on the release of the 40 kD protein were observed. The observed parallel between the effects of (anti)-androgens on the growth of the LNCaP prostate cells and the release of the 40 kD protein suggests that this protein is involved in the regulation of malignant prostate cell growth.

FOOTNOTES

Abbreviations used: AR, androgen receptor; CA, cyproterone acetate; LNCaP, Lymph node carcinoma of the prostate; FGC, fast growing colony; R1881, 17β-hydroxy-17α-methyl-(^{3}H)-estra-4,9,11-trien-3-one; Triamcinolone acetonide, 9α-fluoro-11β,16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide; RU 23908, 5,5-dimethyl-3-(4-nitro-3-(trifluoromethyl)-phenyl)-2,4-imidasolidinedione); R5020, 17α,21-dimethyl-19-nor-

pregna-4,9-diene-3,20-dione; RPMI-medium, Roswell Park Memorial Institute-medium.

INTRODUCTION

The rates of differentiation and proliferation of human prostate and mammary carcinoma are dependent upon androgen or estrogen stimuli respectively. The hormonal effects are mediated by intracellular receptors, which after binding the specific steroid interact with regulatory sites on the genome. As a result mRNA synthesis is stimulated and synthesis of proteins is increased. Newly synthesized proteins have received considerable interest as potential (hormonally regulated) specific tumor markers, and have been studied thoroughly for mammary tumor cells (1-4). For example, in the mammary tumor cell line MCF-7 synthesis of a 24 kD intracellular protein (5) and of the 52 kD released glycoprotein (2) is stimulated by estrogens and inhibited by anti-estrogens. In recent immunocytochemical studies with both tumor and non-malignant tissues it has been suggested that the 52 kD protein, but not the 24 kD protein, is a tumor specific protein (6,7,8). The 52 kD protein is released from tumor cells in the culture medium and may act as an autocrine growth factor (9), since conditioned medium from estrogen stimulated MCF-7 cells, as well as the purified glycoprotein stimulates the growth of the MCF-7 cells which have not been treated with estrogens (10).

In a comparable way androgen regulated growth factors may be involved in the growth of androgen responsive tumor cells. Although several human prostatic tumor lines such as Honda (11), LNCaP (12), PC-3 (13) and PC-82 (14) are available, only the LNCaP (Lymph node carcinoma of the prostate) cell line shows both hormone dependency and continuous growth in vitro. In addition the LNCaP cells produce prostate specific acid phosphatase, and contain androgen receptors (15). This LNCaP cell line seems, therefore, to be a good model for study of possible autocrine or paracrine regulatory growth mechanisms in androgen-dependent prostate tumors.

In this paper we show the effects of androgens and anti-androgens on the cell growth of a fast growing colony of the LNCaP cell line (LNCaP-FGC), and we present evidence for androgen-dependent protein release from these cells in vitro.

MATERIALS AND METHODS

Steroids

5α-Dihydro-(1,2,4,5,6,7,-^{3}H)-testosterone (SA:145 Ci/mmol), 17β-hydroxy-(17α-methyl-^{3}H-estra-4,9,11-trien-3-one (R1881, SA: 87 Ci/mmol), 17β-(2,4,6,7-^{3}H)-estradiol (SA: 99 Ci/mmol) and (^{3}H)-R5020 17α-methyl-^{3}H-,21-methyl-19-nor-pregna-4,9-diene-3,10-dione (R5020, SA: 86 Ci/mmol) and unlabelled R5020 and R1881 were obtained from New England Nuclear (Boston, MA). Unlabelled estradiol, 5α-dihydrotestosterone (DHT), triamcinolone acetonide, cortisol, dexamethasone and progesterone were purchased from Steraloids (Pawling, NY). Cyproterone acetate was a gift of Schering (Berlin, F.R.G.). RU 23908 was a gift from Roussel Uclaf (Paris, France). The radiochemical purity of the labelled steroids verified by thin layer chromatography was greater than 95%.

Cell culture

The LNCaP-FGC cell line (derived from a fast growing colony of the lymph node carcinoma of the prostate) was a gift of Dr. Horoszewicz (Buffalo, NY). The cells were cultured at 37°C in RPMI-1640 medium, with added glutamine, supplemented with 15% (v/v) heat inactivated (single-lot no. 29044106) fetal calf serum (FCS), obtained from Flow laboratories, (Irvine, U.K.) 10,000 IU penicillin and 10,000 μg streptomycin per liter medium in a humidified atmosphere of 5% CO_2 in air. Cells were trypsinized once a week and the culture medium was changed once a week. LNCaP-FGC cells between the 28th and the 61th passage in vitro were used for the studies in this report.

Hormonal sensitivity

Cells were dispersed with trypsin and plated out at a cell density of about 0.75-1x10^{6} cells/25 cm^{2} flask (Falcon, Oxnard, CA). After 3 days the medium was removed and new medium containing either 15% FCS or 15% stripped FCS (obtained by treatment twice with dextran-coated charcoal

(dextran, 0.1%; charcoal, 1%) containing the indicated hormone were counted using a Bürker-chamber.

Assay of (^{3}H)-dihydrotestosterone and (^{3}H)-R1881 metabolism

Cells were incubated for 5 days in 25 cm^2 Falcon flasks. Cells (approximately $5x10^6$) were washed twice with Ca^{2+}/Mg^{2+}-free phosphate buffered saline (Gibco Europe, Paisley, Scotland). Two ml RPMI-1640 medium containing either 25 nM (^{3}H)-DHT or 25 nM (^{3}H)-R1881 were added to the cultures. Following incubation for 1 h at 37°C, the medium was collected and extracted twice with an equal volume of ethylacetate. The solvent was evaporated to dryness, the residue was dissolved in 50 μl ethylacetate and the samples were applied to silicagel thin layer plates (Merck, Darmstadt, FRG). The plates were developed twice in dichloromethane/ether, 85:15 (v/v). Radioactivity was detected with a Panax (Redhill, U.K.) thin layer scanner.

Steroid hormone receptor analysis

Androgen, estrogen and progesterone receptors were measured in the cytosol and in the nuclear extracts of the cells as described by Berns et al. (16,17) and Zava et al. (18). To measure androgen receptors LNCaP-FGC cells (about $8x10^6$) were incubated with 10 nM (^{3}H)-R1881 plus a 500-fold molar excess unlabelled triamcinolone acetonide in the absence or in the presence of a 100-fold molar excess unlabelled R1881. Androgen receptors were also measured by incubation with 25 nM (^{3}H)-DHT in the presence or the absence of a 100-fold molar excess of unlabelled DHT. To determine estrogen receptors cells were labelled with 10 nM (^{3}H)-estradiol in the absence or presence of a 100-fold molar excess of unlabelled estradiol. For progesterone receptors the cells were labelled with 20 nM (^{3}H)-R5020 plus a 100-fold molar excess of unlabelled DHT in the absence or in the presence of a 100-fold molar excess of unlabelled R5020. The cells were incubated with the steroids for 1 h at 37°C in culture medium without FCS, washed with medium and homogenized in TEDG buffer (10 nM Tris-HCl buffer, 1.5 nM EDTA; 1.5 nM dithiotreitol and 10% glycerol (v/v) pH 7.4) to isolate nuclei. The nuclei were extracted with TEDG buffer (pH 8.5) containing 0.4 M KCl and the extract (1.7-2.2 mg

protein/ml nuclear extract) was layered on a sucrose gradient (5-20% sucrose) containing 0.4 M KCl. Cytosol was prepared as described by Berns et al.(16,17). Cytosol was layered also on sucrose density gradients. After centrifugation at 1°C for 23 h at 300,000 g_{av} fractions were collected and counted.

To examine the DNA-binding properties of the extracted androgen receptor, an aliquot of the nuclear extract was desalted on a Sephadex-G25-M column (Pharmacia, Bromma, Sweden). This fraction was incubated for 2 h at 0°C with DNA-cellulose. After washing the column, bound receptor was eluted from the DNA-cellulose with 0.4 M KCl and the eluate was analyzed by sucrose gradient centrifugation as described above. The Stokes radius (R_s) of the receptor was analyzed using Ultrogel ACA34 gel filtration as described by De Boer et al. (19). Protein was determined as described by Bradford (20). Estrogen receptor measurement in LNCaP-FGC cells was also performed with an immunocytochemical assay for the detection of human estrogen receptor as described by the manufacturer (Abbott Laboratories, Chicago, Illinois) (21).

Labelling and analysis of proteins

Cells were trypsinized and plated out in multi well plates (5 mm diameter), in medium containing 15% FCS. After 3 days medium was aspirated and medium containing 15% FCS or medium with stripped 15% FCS was added. The media were renewed after two days. After another two day period, the cells were cultured for two 24 h periods in medium containing stripped FCS plus the hormone to be tested with a medium change after 24 h. Subsequently, the cells were labelled for 6 h in 100 µl of RPMI-1640, with one tenth of the normal concentration of methionine, containing 20 µCi (^{35}S)-methionine (SA: 1096.5 Ci/mmol) (NEN, Boston, MA), as described by Westley and Rochefort (2). Media were collected, centrifuged at 1000 g for 4 min and the supernatants were stored frozen at -20°C until use. Cells were lysed in 100 µl hot lysis buffer and kept frozen at -20°C until use. Aliquots of 50 µl of the cell lysate or a volume of medium with approximately 0.03 µCi of (^{35}S)-labelled methionine (incorporated into proteins and residual methionine), were analyzed by one dimensional SDS-PAGE (11% linear gel), fluorographed and exposed as indicated in the legends to the figures, using SB-5 films

(Kodak, Rochester, N.Y.).

RESULTS

Hormone sensitivity

The growth and function of the prostate are primarily dependent on androgenic stimuli (22). To investigate whether the human prostate epithelial cell line LNCaP-FGC is responsive to male sex hormones, we have examined the effect of androgens on the in vitro growth of these cells. Since fetal calf serum (FCS) contains small amounts of androgens (approximately 1 nM testosterone), we have studied the growth of the LNCaP-FGC cells in 15% FCS in the absence and presence of an anti-androgen, which blocks androgen action and in "stripped" 15% FCS from which steroids were removed by charcoal adsorption (less than 0.15 nM testosterone).

Cells cultured with 15% FCS showed a mean population doubling time of about 73 hours. Addition of androgens to this medium hardly affected the growth rate. In contrast, the anti-androgen cyproterone acetate suppresses cell growth to 50% of that obtained in complete 15% FCS (Figure 1). Hence, this suggests that androgens already present in the FCS stimulate the cell growth. To investigate this we have cultured the cells in 15% stripped

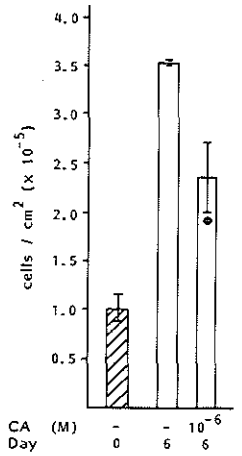

Figure 1. Effect of 15% fetal calf serum and cyproterone acetate (CA) on the proliferation of LNCaP-FGC cells in culture.

LNCaP-FGC cells were seeded at a cell density of 0.75 - 1 x 10^6 cells/flask in medium RPMI-1640 with 15% (v/v) fetal calf serum. Following attachment (3 days), the number of cells was counted (D=0). Cells were further incubated either with or without 1 μM cyproterone acetate (CA). Media were changed every other day and after six days the number of cells was counted. Cells in different groups were incubated in triplicate. The effect of CA was significant (•; $p<0.01$).

serum. Under these conditions a slower growth rate with a mean population doubling time of approximately 140 hours was measured. To analyse the cell response to androgens, we have added various concentrations of 5 -dihydrotestosterone (DHT, 0.01 - 1 μM) to media with stripped serum. DHT slightly increased the cell growth (not shown). An explanation for this minor effect on cell growth might be the rapid metabolism of DHT (described in the next section). We have therefore studied the effect of the non-metabolizable androgen R1881 on the proliferation rate. This synthetic androgen has a high affinity for both androgen and progesterone receptors (23), but progesterone receptors are absent in the LNCaP-FGC cells (see receptor section). Any effect of this steroid must therefore be ascribed to its androgenic properties. A dose-dependent stimulation of cell growth was observed when R1881 (0.01 - 100 nM) was added to the stripped FCS, resulting in a maximal stimulation factor of 2.3 when 0.1 nM R1881 was added. Higher doses of R1881 were less stimulatory (Figure 2). When

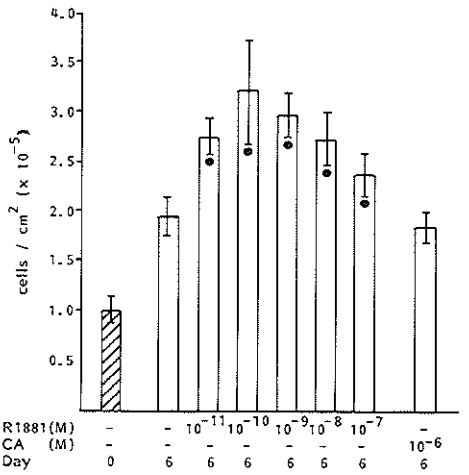

Figure 2. Effect of different concentrations of R1881 and cyproterone acetate (CA) on the proliferation of LNCaP-FGC cells in culture medium with 15% stripped serum.

LNCaP-FGC cells were seeded at a cell density of 0.75 - 1 x 10^6 cells/flask and grown for 3 days. After 3 days, the number of cells was counted (D=0). Cells were further grown in stripped serum with different concentrations of R1881 or with cyproterone acetate (CA). Medium was changed every other day and the cells were counted after six days. Cells in different groups were incubated in triplicate. Significant differences ($p<0.05$) with control values after 6 days of culture are indicated as: ●.

cyproterone acetate (1 μM) was added to the stripped serum, no significant change in cell growth was observed, indicating that stripping efficiently removes androgens from the serum.

From the results described above we have concluded that the growth of the LNCaP-FGC cell line can be regulated by the addition of exogenous androgens.

Metabolism of androgens

In the study on hormonal sensitivity we observed that the non-metabolizable androgen R1881 had a greater effect on cell growth than the natural androgen DHT. Hence, we have studied the metabolism of the two different androgens by the LNCaP-FGC cell line in culture. Cells were incubated for 1 h at 37°C with (^{3}H)-DHT or (^{3}H)-R1881. After incubation the media were analyzed for the presence of non-polar and polar metabolites (Table 1). Approximately 60% of the (^{3}H)-DHT was converted to polar metabolites since this amount could not be extracted with ethylacetate. The ethylacetate extract contained approximately 80%

Table 1. Metabolism of androgens by LNCaP-FGC cells.

Steroid	incubation time (h)	% polar degradation products	composition of non-polar steroids
DHT	0	5%	89% DHT
DHT	1	61%	80% DHT; 4% androstanedione
R1881	0	4%	100% R1881
R1881	1	5%	100% R1881

Metabolism of (^{3}H)-DHT and (^{3}H)-R1881 by the LNCaP-FGC cells in culture. Two ml RPMI-1640 medium containing either 25 nM (^{3}H)-DHT or 25 nM (^{3}H)-R1881 were added to the cultures. Media were analysed for the presence of polar and non-polar metabolites, before and after incubation with the labelled steroids for 1 h at 37°C. Aliquots were extracted with ethylacetate to separate polar and non-polar steroids. The percentage of polar degradation products is expressed as percentage of the added (^{3}H)-steroid. Ethylacetate extracts were evaporated to dryness and analysed by thin layer chromatography. The composition of steroids is expressed as percentage of extracted (^{3}H)-steroid.

intact DHT and 4% androstenedione. We have calculated that (^{3}H)-DHT is rapidly metabolized at an approximate rate of 12.5 pmol/h/5x10^6 cells. As a result 50% of the added (25 nM) DHT is metabolized within 2 hours. In contrast (^{3}H)-R1881 is not converted to a polar metabolite. The corresponding ethylacetate extract contained 100% intact labelled steroid. In view of these findings we have performed most of the subsequent studies with the non-metabolizable synthetic androgen R1881.

Receptors

The sensitivity of the LNCaP-FGC cells for androgens makes it likely that specific receptors for androgens are present. We have therefore measured androgen receptors by incubating the cells with (^{3}H)-R1881. To avoid possible binding of R1881 to the progesterone receptor, the cells were incubated with a 500 molar excess of triamcinolone acetonide, as described by Zava et al. (18). In the nuclear extract a mean value of 1,679 ± 558 (mean ± S.D., five determinations) fmol androgen receptor/mg nuclear extract protein was obtained. This corresponds to 17,000 ± 2,500 (mean ± S.D.) sites/cell. Sucrose gradient centrifugation in 0.4 M KCl revealed a peak sedimenting at 4.5S (4.5 ± 0.2; mean ± S.D., eleven determinations) and a shoulder at 3S (2.8 ± 0.3; mean ± S.D., nine determinations) (Figure 3A).

Gelchromatography on an Ultrogel ACA34 column gave a Stokes radius of about 4.7 nm for the entity sedimenting at 4.5S. A molecular mass of 91 kD can be calculated from these data (19). When a nuclear extract was left on ice for about 4 h, the sucrose gradient profile revealed two receptor peaks, one sedimenting at 4.5S and the other sedimenting at 3S (Figure 3B). The latter form corresponds to a molecule with Stokes radius of 2.7 nm by gelchromatography and a molecular mass of approximately 33 kD was calculated. The appearance of this small, 3S, form may reflect the presence of proteolytic enzyme activity in the nuclear extract.

The androgen receptor content in the cytosol (4-4.2 mg protein/ml cytosol) of the LNCaP-FGC cells was also measured after culture in charcoal-stripped serum containing medium. In the cytosol of the LNCaP-FGC cells approximately 900 fmol androgen receptor/mg cytosol protein are present, corresponding to 23,000 sites/cell, a number of sites comparable to the number found in the nuclear extract after incubation of cells with

R1881. Sucrose gradient centrifugation in 0.4 M KCl revealed a peak sedimenting at 3.6 S (not shown).

To examine the DNA-binding properties of the two receptor forms identified in the nuclear extract, aliquots were desalted and allowed to

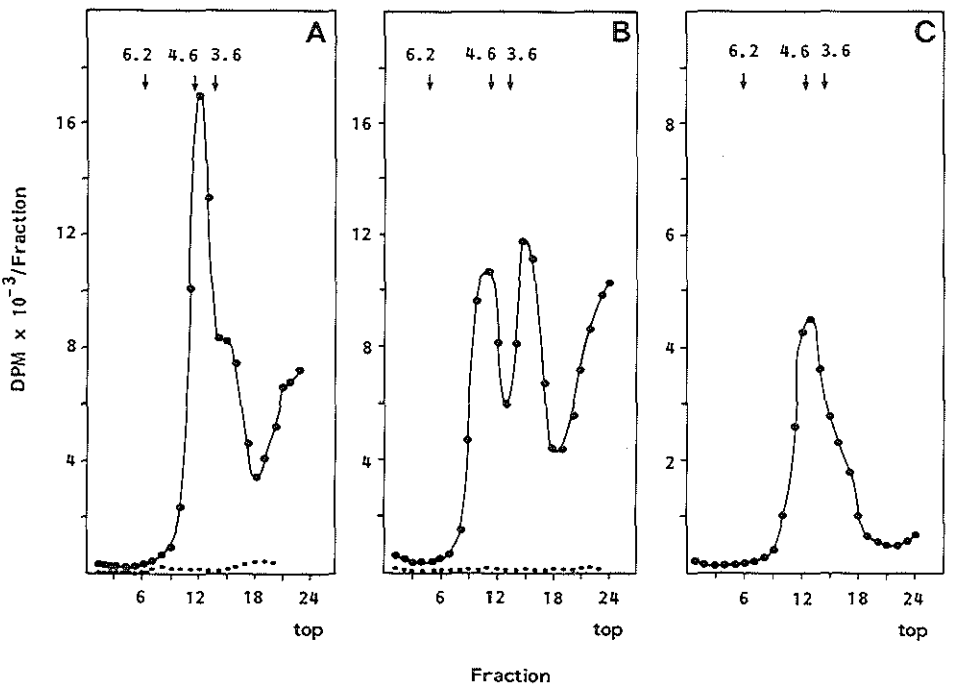

Figure 3. Sucrose density gradient analysis of the androgen receptor in the nuclear extract of LNCaP-FGC cells.
A) Cells were incubated for 1 h with 10 nM (^{3}H)-R1881 with 500 nM triamcinolone acetonide in the absence (●—●) or in the presence (● ●) of a 100 fold molar excess of unlabelled R1881. The nuclear extract was analysed on a sucrose density gradient as described in the methods section.
B) A nuclear extract, prepared as in panel A) was kept at 4°C for 4 h and analysed on a sucrose density gradient as decribed in the methods section.
C) A nuclear extract, prepared as in panel A), was desalted and bound to DNA cellulose as described in the methods section. Bound receptor was eluted with 0.4 M KCl and analysed on a sucrose density gradient. Alkaline phosphatase (6.2 S), bovine serum albumin (4.6 S) and ovalbumin (3.6 S) were used as sedimentation markers.

interact with DNA cellulose. On sucrose gradients in 0.4 M KCl the bulk of the androgen receptor eluted from the DNA cellulose sedimented at 4.5S (Figure 3C), indicating that the 4.5S form was the predominant DNA-binding form. The small fraction sedimenting at 3S is probably generated as a result of residual protease action. We have also screened the LNCaP-FGC cell line for the presence of estrogen and progesterone receptors. In two separate experiments estrogen and progestin receptors were not detectable in the nuclear extracts from cells incubated with (^{3}H)-estradiol or (^{3}H)-R5020 respectively. The detection limit of the sucrose gradient assay was about 15 fmol/mg protein. Estrogen and progesterone receptors were also not detectable in the cytosol of these cells. In addition, we have applied an immunocytochemical assay with monoclonal antibodies directed against the estrogen receptor from MCF-7 cells. Also with this assay, estrogen receptors could not be measured in the LNCaP-FGC cells. The detection limit of the immunocytochemical assay was about 20 fmol/mg protein.

Hormone regulated proteins

To examine a possible effect of androgens on specific proteins, cells were labelled with (^{35}S)-methionine after culturing the cells in the presence of different steroid hormones. Cells or media were collected and the radioactive proteins were separated by SDS-polyacrylamide gelelectrophoresis, fluorographed and exposed. Figure 4 shows that the patterns of intracellular proteins after 9 days of culture in medium containing either stripped FCS or complete FCS were identical. In contrast, a protein with a relative molecular mass of 40 kD was released from cells grown in normal serum and had completely disappeared when the cells were cultured for four days in stripped serum. This 40 kD protein was still absent when the cells were cultured for another 4 days in stripped FCS. In the presence of DHT (0.1 - 1 μM) or in the presence of the non-metabolizable androgen R1881 (0.1 - 100 nM) the release of the 40 kD protein in the medium was partially restored. Lower concentrations of DHT did not have a significant effect on the release of the 40 kD protein. This is probably related to the rapid metabolism of DHT by the LNCaP-FGC cells.

As reported above the steroidal anti-androgen cyproterone acetate

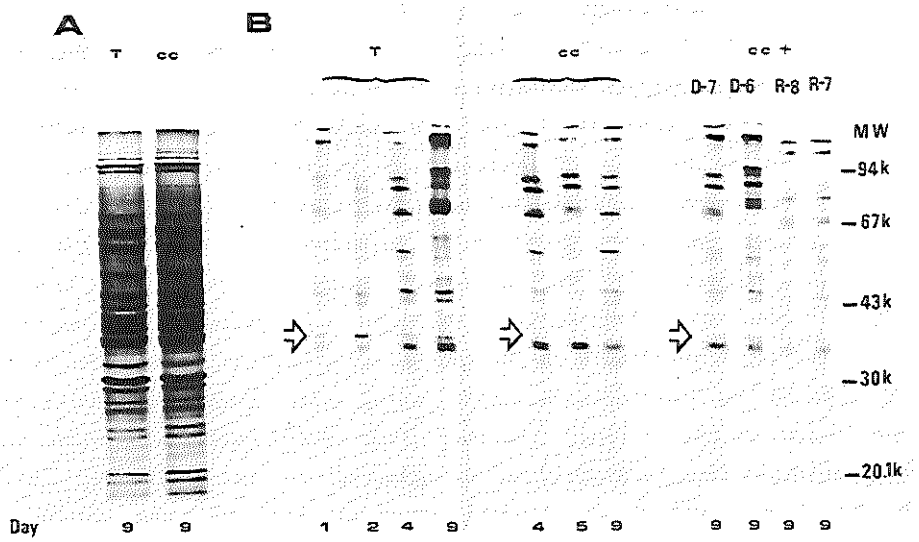

Figure 4. SDS-polyacrylamide gels (11%) of cellular (A) and released proteins (B) synthesized under different conditions by LNCaP-FGC cells. Cells were cultured for various time periods in medium containing normal serum (T), stripped serum (cc) and stripped serum with added 10 nM and 100 nM R1881 (R-8; R-7) or 0.1 M and 1 μM DHT (D-7; D-6). Steroids were added to the stripped serum on days 7 and 8. At the end of the culture period, the cells were labelled for 6 h with (^{35}S)-methionine and the secreted and cellular proteins were analysed by SDS-PAGE and fluorography. Panel A (cellular proteins) containes 200.000 cpm/lane and panel B (released proteins) containes 17.000 cpm/lane. The arrow indicates the position of the 40 kD released protein.

inhibits the growth of the LNCaP-FGC cells. To investigate a possible relationship between cell growth and the release of the 40 kD protein we have also studied the effects of anti-androgens on the release of this protein. The anti-androgens were added in a large excess (varying from 100 to 10,000 fold molar excess), because their affinity for the androgen receptor is low when compared to the affinity of DHT or R1881 for this receptor. Addition of cyproterone acetate in a 100 to 10,000 fold molar excess partly inhibited the R1881 induced release of the 40 kD protein (Figure 5). RU 23908, a non-steroidal anti-androgen, also reduced the R1881 induced release of the 40 kD protein when added in a 1000 fold molar

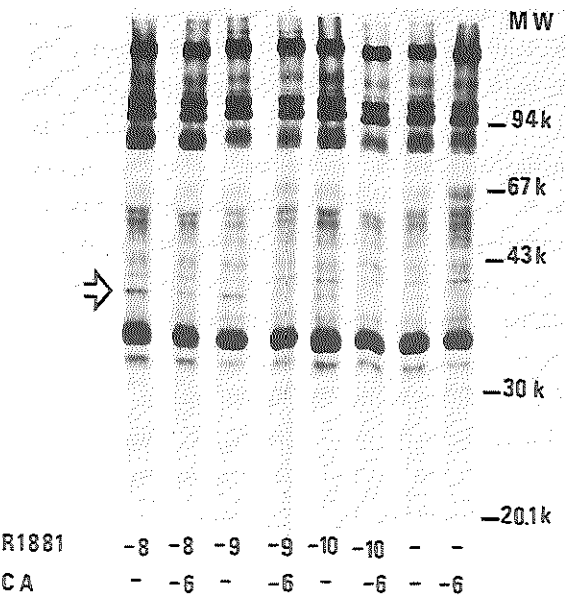

Figure 5. Effects of cyproterone acetate (CA) on the release of the 40 kD protein. Cells grown for 4 days in 15% stripped serum, were further cultured for 48 h in medium with 15% stripped serum containing varying concentrations of R1881 (0.1-10 nM) without or with cyproterone acetate (CA) (1 μM). Subsequently proteins were labelled for 6 h with (^{35}S)-methionine and the media were analysed on SDS-PAGE and fluorography. The arrow indicates the position of the 40 kD released protein.

excess (not shown).

The release of this protein is not stimulated by estrogens, progesterone or glucocorticoids (cortisol and dexamethasone). Hence the release of the 40 kD protein is specific for androgens.

DISCUSSION

The characteristics of the LNCaP cell as described by Horoszewicz et al. (15) make this cell attractive as a model for the study of human prostate cancer in vitro: the cells have been found to be hormone-responsive,

produce prostate acid phosphatase (a long recognized marker of prostatic cancer) and contain androgen receptors. We have used a fast growing colony from this LNCaP cell line, designated LNCaP-FGC and observed that growth of these cells is also stimulated by androgens (2.3 times maximally with 0.1 nM of the non-metabolizable androgen R1881), whereas anti-androgens were suppressive. The nuclear extract of the LNCaP-FGC subline contained a large number of androgen receptors, corresponding to approximately 17,000 sites per cell. Based on these observations we have concluded that our LNCaP-FGC cells, like the original LNCaP cells are androgen-sensitive.

In the original report describing the LNCaP cells, Horoszewicz et al. (15) showed 1.9-fold stimulation of cell growth by dihydrotestosterone and a receptor content in the cells of approximately 210 fmol androgen receptor/mg cytosol protein. In contrast, Hasenson et al. (24) only observed a weak positive effect on the mitotic index when 10-100 nM dihydrotestosterone was added to the cells and found a low number of androgen receptors in these cells, approximately 16 fmol androgen receptor/mg protein. Consequently these authors concluded that their LNCaP cells were androgen resistant and designated this cell line LNCaP-r. The discrepancies in hormonal responsiveness observed in the different studies may be due to variation in the amount of androgen receptor in the different sublines of LNCaP which were used. Another explanation for the observed discrepancies in stimulation of cell growth may be found in the rapid metabolism of the DHT added to the culture media (15, 24) and consequently a longer lasting effect of the non-metabolizable androgen R1881 was measured in our studies. In addition the use of stripped fetal calf serum (with a low endogenous androgen level) in our studies enhances response to androgens. Apart from these hormonal effects, other factors can not be excluded, since Metcalfe et al. (25) have shown that cell growth of LNCaP cells is also dependent on the number of cells plated.

In this report we have described two different forms of nuclear androgen receptors in the LNCaP-FGC cells. The larger 4.5S form represents the DNA-binding form and is converted in a time dependent process into a smaller 3S form. The small 3S form is generally found in extracts from tissues with high proteolytic activity and the larger form in extracts from tissues with relative low endogenous proteolytic activity (19, 26,

27). Our results with the LNCaP-FGC cell line therefore suggest that the 3S form is an artifact of preparation due to considerable protease activity in the nuclear extracts.

Estrogen receptors could not be detected in the nuclei or cytosol of the cells with either a biochemical or an immunocytochemical assay. In contrast to these results, Horoszewicz et al. (15) have found estrogen binding proteins in cytosol preparations of the LNCaP cell with a somewhat higher Kd than usually observed for estrogen receptors in target tissues (5.2 nM, compared to a normal range of 0.1 - 2 nM). This discrepancy needs further investigation.

Since androgens stimulate and anti-androgens suppress the cell growth of the LNCaP-FGC cells, we have investigated the effects of these steroids on specific protein synthesis and specific protein release. Although we did not observe any apparent differences in the intracellular protein patterns, the addition of androgens did change the release of some proteins into the medium and especially increased the release of a protein with a relative molecular mass of 40 kD. The release of this protein was not detectable when the cells were grown in stripped fetal calf serum with a low endogenous androgen level. After addition of R1881 (0.1 nM to 0.1 μM) or DHT (0.1 to 1 μM) the release of the 40 kD protein was restored. The relative high concentration of DHT required to obtain this effect probably reflects the rapid metabolism of DHT, as had been found for this steroid in the present studies. Addition of the anti-androgens cyproterone acetate or RU 23908 together with androgens also reduced the amount of released proteins. Because these anti-androgens have a low relative binding affinity for the androgen receptor (28, 29), it was necessary to add a 100 to 10,000 molar excess of these steroids to obtain an inhibitory effect on protein secretion. The stimulatory effect on the release of the 40 kD protein is apparently specific for androgens, since no effect on release was found for either estrogens, progestins and corticoids, which is in agreement with the absence of receptors for these steroids.

To our knowledge, this is the first evidence for a stimulatory effect of androgens on the release of a specific protein by human prostatic tumor cells in culture. For the T47D human breast cancer cell line the synthesis of androgen-regulated proteins with unknown function of 43, 22 and 18 kD

has recently been described and the production of the 43 kD protein was also inhibited by anti-androgens (30). In this respect it is of interest to mention the studies of Edwards et al. (31), who have identified a series of proteins, designated PCA-1, in the urine of patients with prostate cancer. These PCA-1 proteins with a molecular mass of approximately 40 kD could only be detected in urine of patients with prostatic cancer and not in the urine of patients with other non-prostatic cancers, benign prostate hypertrophy or normal men. It is therefore of interest to study a possible relationship between the androgen induced 40 kD protein released by LNCaP-FGC cells and the prostate cancer 40 kD protein.

The function of the 40 kD protein released by the LNCaP cells has not been studied. An intriguing possibility is that this protein functions as an autocrine growth factor for prostate cells. Such a function has been shown for a 52 kD glycoprotein which is released from MCF-7 mammary tumor cells after growth stimulation by estrogens (10). After purification this protein was able to stimulate MCF-7 cell growth in a similar way as after addition of estrogens to the cells.

Further studies on the isolation and purification of the 40 kD protein may be relevant to obtain better markers of hormonal responsiveness for tumor cell growth and may eventually open new possibilities for the therapy of prostatic carcinoma.

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