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Human prostate cancer: a direct role for oestrogens

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Abstract. We have studied the response to oestrogen and expression of oestrogen receptors in responsive LNCaP and androgen non-responsive PC3 human prostate cancer cell lines. Growth of LNCaP cells is significantly stimulated by physiological concentrations of oestradiol; this growth increase appears to be comparable to that induced by either testosterone or dihydrotestosterone. In contrast, oestradiol significantly inhibits the proliferation of PC3 cells. We also present novel evidence for functional oestrogen binding in LNCaP cells. This evidence was first obtained by means of radioligand binding assays and was further corroborated using: (a) immunocytochemical analysis of oestrogen and progesterone receptors; (b) reverse transcriptase polymerase chain reaction of oestrogen receptor mRNAs; and (c) immunofluorescence of the 27 kDa heat shock protein (Hsp27), which has been reported to be a marker of functional oestrogen receptors. There appeared to be significantly and consistently lower levels of oestrogen receptor expressed in PC3 cells than in LNCaP cells. The observation that oestradiol-induced growth of LNCaP cells is completely reversed by the pure anti-oestrogen ICI 182 780 clearly implies that the biological response of these cells to oestradiol is mediated mainly via its own receptor. On the other hand, use of a neutralizing antibody against transforming growth factor (TGF)-β1 results in a remarkable increase in the growth of PC3 cells; this effect is almost completely abolished after the addition of oestradiol. This suggests that the oestradiol-induced growth inhibition may be mediated by TGF-β1. These results suggest that the current model for hormone-dependence of human prostatic carcinoma should be revised. This is of special concern, because recent data indicate that prostate cancer has become the most prevalent cancer and the second principal cause of cancer death in western countries.

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The incidence of prostate cancer varies greatly throughout the world; it is highest in African–Americans and lowest in the Asian populations of China and India (Zaridze et al 1984). Overall, statistical data indicate that during the last decade its incidence has been rising dramatically worldwide, to such a level

that prostate cancer has become the most prevalent cancer and the second principal cause of cancer death (Carter & Coffey 1990). The most recent epidemiological studies give an alarming indication of the dimensions of the problem: in the USA, more than 200 000 new cases of prostatic cancer are to be expected in 1994, resulting in approximately 58 000 deaths (Garnick 1994).

Autopsy studies have revealed that the human prostate has a uniquely high occurrence of latent microcarcinomas, the latter being associated with the mortality rates of clinically apparent prostate cancer (Breslow et al 1977). It has been postulated that tumour initiation does not differ in populations having high or low mortality rates, but that geographical differences in both prevalence of latent prostatic cancer and mortality can be ascribed to the influence of diverse tumour-promoting factors, either environmental or dietary.

It is a widely held belief that human prostate cancer is essentially androgen dependent. This assumption has influenced strategies for endocrine therapy (including total androgen blockade) of prostate cancers. In contrast, experimental results from animal model systems have shown that long-term treatment with testosterone and/or oestradiol, but not dihydrotestosterone, results in the development of prostatic tumours (Leav et al 1978). This has led to the hypothesis of conjoint, androgen-supported oestrogen-enhanced induction of aberrant prostate growth (Leav et al 1989). It is worth noting that pharmacological doses of synthetic oestrogens, such as diethylstilbestrol (DES), have proved to be an effective therapy for advanced human prostatic cancer. The high response rates (70–80%) obtained in prostate cancer patients have been ascribed merely to the fact that, following DES administration, plasma levels of testosterone drop to those found in castrates (Turkes et al 1988). The latter, however, have been argued to be still compatible with the maintenance of malignant prostate growth (Labrie et al 1993). In addition, intravenous stilbestrol diphosphate, and oestrogens in general, have been reported to be beneficial even in patients with metastatic, hormone-refractory disease (Ferro 1991).

In vitro systems are frequently used as models for most hormone-dependent tumours, including those of the prostate. We have investigated both androgen-responsive LNCaP (Fig. 1A) and androgen-unresponsive PC3 (Fig. 1B) human prostate cancer cell lines to gain insight into mechanisms of oestrogen growth control in these systems.

Expression of oestrogen-binding proteins and mRNA transcripts

High-affinity sites of oestrogen binding

Several studies have explored the effects and the mechanisms of action of oestradiol and other oestrogen-related steroids in the growth regulation of

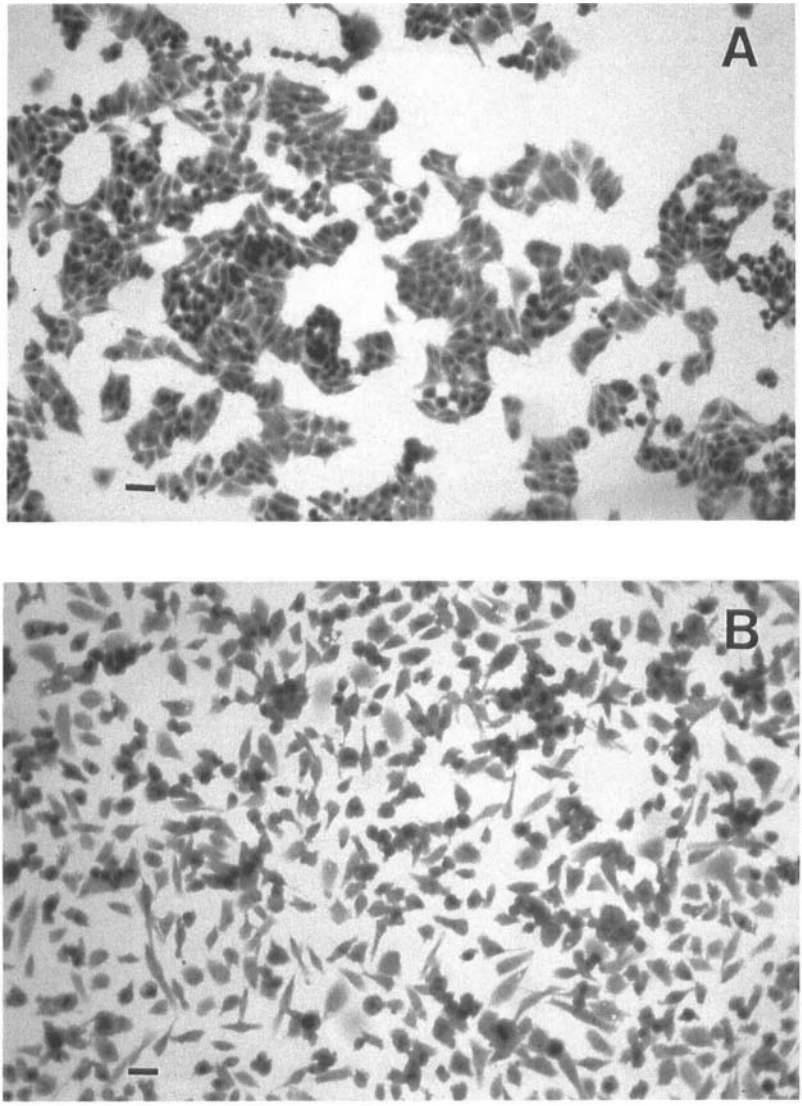

FIG. 1. Photomicrographs of (A) LNCaP and (B) PC3 cells in culture (bar = 500 μm; Papanicolau staining).

androgen-responsive LNCaP human prostate cancer cells. Investigators have mainly used radioligand binding assays to measure either the androgen or the oestrogen receptor content of this cell line.

Originally, Horoszewicz et al (1983) revealed the presence of oestrogen-binding sites in the cytosolic fraction of LNCaP cells, with dissociation

TABLE 1 Studies on androgen receptor status and relative binding affinity (RBA) for oestradiol in LNCaP human prostate cancer cells

Author	*Cell fraction assayed*	*Radioligand concentration (nM)*	*Dissociation constant (nM)*	*Concentration (fmol/mg P)*	*RBA for oestradiol*[a]
Berns et al 1986[b]	cytosol nuclear extract	10 R1881 25 DHT	NR	900 1679	ND
Schuurmans et al 1988	cytosol	0.5–10	0.4	920	4.3
Sonnenschein et al 1989	whole cell extracts	2–20	3.0	68	0.4
Veldscholte et al 1990b	cytosol	0.5–10	0.4	NR	2.4
Kirschenbaum et al 1993	whole cells	0.3–6	3.1	1366	6.0

[a]RBA was expressed (in percentage) as the ratio of amounts of unlabelled R1881 and competing oestradiol which are required to inhibit binding of tritiated R1881 by 50%. The RBA for R1881 was set as 100%.
[b]Single point assay.
P, protein; NR, not reported; ND, not determined.

constant (K_d) values in the nanomolar range (nearly 5 nM). Further studies, however, failed to detect oestrogen receptor in either nuclear extract or soluble fraction of LNCaP cells (Berns et al 1986, Sonnenschein et al 1989). Parallel work has indicated that LNCaP cells contain one point mutation in codon 868 of the androgen receptor gene; this mutation (A to G) results in the transition of a threonine into an alanine residue at the C-terminus of the steroid-binding domain of the receptor (Trapman et al 1990). It has been suggested that this abnormality confers to the androgen receptor system a broader steroid specificity, allowing binding of oestrogens, progestogens and several anti-androgens, and the resulting induction of gene expression (Veldscholte et al 1990a). Results from relevant studies are summarized in Table 1. As can be seen, the relative binding affinity (RBA) reported for oestradiol ranged from 0.4–6.0% of that of the synthetic androgen R1881. It must be emphasized that most of these studies used ligand concentrations (sometimes even as a single point assay), which do not permit the proper identification of high-affinity, low-capacity (type I) binding sites, but instead mostly involve type II sites, which have higher K_d values (in the nanomolar range). In keeping with the original definition of the biochemical and functional features relevant to distinct sites of oestrogen binding (Clark & Peck 1979), this may represent a critical shortcoming, especially in the light of the fact that only the type I oestrogen receptors have been shown to be of value in the prognosis of and for the

TABLE 2 Status of soluble and nuclear Type I and Type II oestrogen receptors in LNCaP cells

	Soluble fraction			*Nuclear fraction*		
	K_d *(nM)*	*fmol DNA*	*Sites/cell*	K_d *(nM)*	*fmol DNA*	*Sites/cell*
Type I	0.49 ± 0.03	2124 ± 368	79 755 ± 4603	0.25 ± 0.09	328.1 ± 48.2	13 737 ± 2006
Type II	7.24 ± 0.30	11 785 ± 3867	442 520 ± 48 369	7.02 ± 3.51	1942 ± 513	81 308 ± 21 350

Oestrogen receptor content and status of LNCaP cells was assessed in both soluble and nuclear compartments through radioligand binding assays (for details of methodology see Carruba et al 1993). Values represent means ± SD of $n = 7$ assays.

response to endocrine treatment of breast and endometrial cancer patients (Castagnetta et al 1987a, 1992).

We have recently investigated the presence of oestrogen-binding sites in both LNCaP and PC3 cells by radioligand binding assay, using a ligand ([^{3}H]oestradiol) concentration range of 0.1–5 nM (precisely, 0.1, 0.2, 0.3, 0.5, 0.75, 1, 2, 3 and 5 nM) and a 100-fold excess of unlabelled DES or R1881 for competition studies. As shown in Table 2, our data clearly indicate that both type I (high affinity, limited capacity) and type II (lower affinity, higher capacity) oestradiol-binding proteins are present in either the cytosolic or nuclear fraction of LNCaP cells and that their levels are comparable to those we have found in other oestrogen-responsive cancer tissues (Castagnetta et al 1987a,b) and cells (Lo Casto et al 1983, Castagnetta et al 1986). Conversely, PC3 cells did not show any detectable oestradiol-binding sites in the soluble fraction, whereas type I oestrogen receptors were found in the nuclear fraction. These had mean K_d and concentration values, respectively, of 0.16 ± 0.04 nM and 118.8 ± 22.3 fmol/mg DNA (mean ± SD, $n = 5$). The results of competition studies in LNCaP cells are worth noting (see Fig. 2). Using a 100-fold excess of R1881, an appreciable increase of bound radioligand was observed in both cell fractions; conversely, DES competed effectively for both soluble and nuclear oestrogen binding, a consistent displacement of [^{3}H] oestradiol being observed (ranges of 36.9–57.0% and 35.5–53.4%, respectively). Surprisingly, when R1881 was used as a competitor, Scatchard analysis revealed a substantial increase in type I oestrogen receptor content in both soluble (over 90%) and nuclear (nearly 30%) fractions of LNCaP cells. This evidence suggests that [^{3}H]oestradiol may partly bind to type II androgen receptors; however, since the addition of excess R1881 displaces this type II androgen binding only, a considerable proportion of labelled oestradiol would be made available for further binding to type I oestrogen receptors. Type II binding sites may serve as

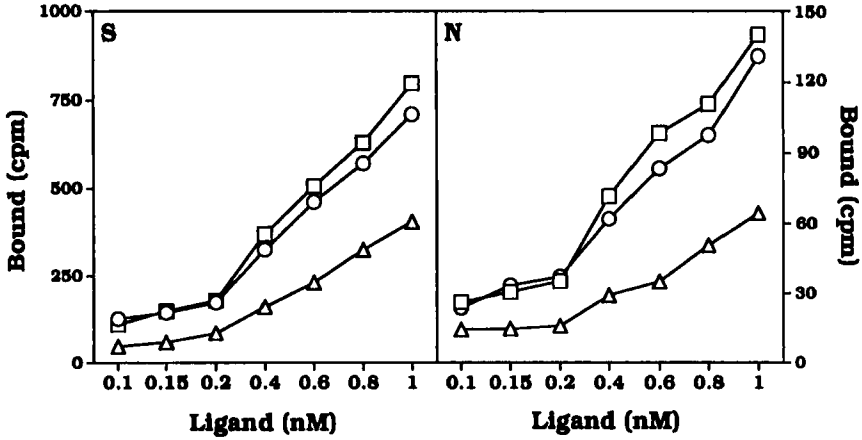

FIG. 2. Ligand binding curves of oestradiol in LNCaP cells. The amount of bound oestradiol was measured through incubation of both soluble (S) and nuclear (N) cell fractions with increasing concentrations of [^{3}H]oestradiol alone (○) and [^{3}H]oestradiol plus a 100-fold excess of the synthetic oestrogen diethylstibesterol (△) or the synthetic androgen R1881 (□). cpm, counts per minute.

storage proteins, which have a reduced affinity and a broader specificity of binding for different steroids (including androgens, oestrogens and progestagens) and antisteroids. Since the conditions currently used for androgen receptor assay are compatible with a prevalent measurement of type II receptors, this may also account for the displacement of androgen binding observed in previous studies using R1881 as the radioligand and oestradiol as the competitor (Sonnenschein et al 1989, Veldscholte et al 1990a, Schuurmans et al 1991, Kirschenbaum et al 1993).

Another source of major concern, which is often mistakenly disregarded, originates from both the conditions used for routine maintenance of cells and the age of the culture. Concerning LNCaP cells, culture conditions vary greatly among several studies, including differences in the culture medium used (RPMI, DME, Ham's F12) and the percentage (5–15%) and treatment (heat-inactivated or not) of fetal calf serum. It is well documented that the parental LNCaP cell line may give rise to several sublines, also depending on steroid addition or depletion, in a relatively short time (van Steenbrugge et al 1991, Kirschenbaum et al 1993). The receptor studies herein discussed have used LNCaP cells which have a wide range of passage number (from the 28th to the 72nd passage *in vitro*), corresponding to between 100 and 400 population doublings after the original isolation. This, apart from the differences in the experimental conditions used, could make it difficult to obtain reliable or comparable results. In our experience, the use of cells at low, narrow-span passages may avoid this problem and limit the risks inherent in *in vitro* systems,

whereby a heterogeneous cell line is invariably exposed to the selective pressures of an otherwise artificial environment.

Quantitative immunocytochemistry of oestrogen and progesterone receptors

Although it is widely recognized that oestradiol stimulates growth of the androgen receptor-positive LNCaP cells, studies on the oestrogen receptor content of this cell line have been surprisingly rare. In a previous report, no oestrogen or progesterone receptors were found in LNCaP cells by either ligand binding or immunocytochemical assays (Berns et al 1986). These authors used monoclonal antibodies directed against the oestogen receptor from MCF-7 human mammary cancer cells but, unfortunately, failed to include details of the methodological approach. More recently, Brolin et al (1992), using the same immunocytochemical assay, reported that both LNCaP and PC3 cells were found to be negative for oestrogen and progesterone receptors.

We have investigated the presence of both oestrogen and progesterone receptors in LNCaP, DU145 and PC3 cells by using modified versions of the commercially available Abbott immunocytochemical assay kits. We analysed the receptor staining using the CAS^{TM}200 image analysis system, which automatically estimates the percentage of positively stained nuclei and measures the intensity of staining; the latter is defined as the summed optical density for the receptor-positive nuclear area over the summed total optical density of all the nuclei, expressed as a percentage. Percentages $\leqslant 30\%$, from 30 to 60% and $>60\%$, respectively, identified weak, moderate and strong intensities of staining. As illustrated in Plate II, LNCaP cells stained intensely for both receptors; the percentages of positive nuclei were 56.7–66.4% and 59.0–72.6%, respectively, for oestrogen and progesterone receptors. The intensity of staining was consistently strong (69.7–82.8% for oestrogen receptor and 79.8–87.9% for progesterone receptor), with a coefficient of variation of $<15\%$. In contrast, PC3 cells displayed very low expression of oestrogen receptors, with about 25% of positive nuclei having a weak-to-moderate degree of staining, and all cells were progesterone receptor-negative. DU145 cells were negative for both oestrogen and progesterone receptors. Our results are at variance with those of Brolin et al (1992). However, this discrepancy could be ascribed simply to the different conditions used for exposure to primary antibodies (1 h at 37 °C as opposed to 24 h at 4 °C in our assay).

It is worth noting that progesterone receptors are commonly thought to associate with functional oestrogen receptors in human breast and endometrial cells; consequently, clinicians have been using their expression as a helpful indicator in the management of breast cancer patients. The presence of progesterone receptors in the LNCaP cell line therefore reinforces the

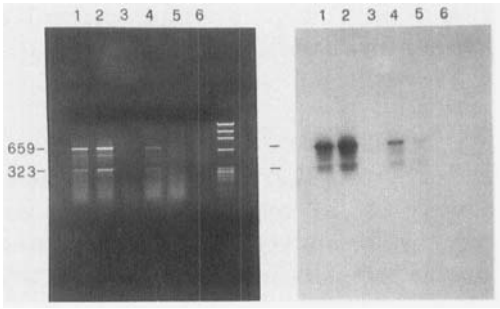

FIG. 3. Reverse transcriptase polymerase chain reaction (RT-PCR) of oestrogen receptors in LNCaP and PC3 prostate cancer cells. (*Left panel*) Ethidium bromide staining of agarose gel electrophoresis of PCR amplification products obtained from one milligram total RNA: lane 1, ZR75-1; lane 2, MCF-7; lane 3, MDA-MB231; lane 4, LNCaP; lane 5, PC3 cells; lane 6, RT-PCR of an RNA-free control sample; marker lane, ϕx-174-*Hae* III digest fragments (length given in bp). (*Right panel*) Southern blot analysis of the PCR-amplified DNA from the left panel. Samples were hybridized using a ^{32}P-labelled human oestrogen receptor cDNA as a probe.

conviction that these cells contain an apparently intact receptor machinery suited to mediate the biological effects of oestrogen.

Reverse transcriptase polymerase chain reaction of oestrogen receptor mRNA transcripts

We investigated the expression of oestrogen receptor mRNAs in human prostate tumour cell lines using reverse transcriptase polymerase chain reaction (RT-PCR) amplification of total RNA extracted from LNCaP, DU145 and PC3 cells; oestrogen receptor-positive ZR75-1 and MCF-7 and oestrogen receptor-negative MDA-MB231 human mammary cancer cell lines were used for comparison. We performed PCR amplification using an oestrogen receptor cDNA (kindly supplied by Pierre Chambon, University of Strasbourg, France) as a template, with a sense primer corresponding to a sequence in exon 3 and an anti-sense primer corresponding to a sequence in exon 6 of the human oestrogen receptor gene. We separated the amplification products on a 1.4% agarose gel and confirmed their identity by Southern blot analysis, using the complete oestrogen receptor cDNA as a probe. As shown in Fig. 3, specific hybridization was seen in mammary ZR75-1 and MCF-7 cells, similar to that in prostate LNCaP and PC3 cells. In particular, two major bands (of 659 and 323 bp) and some minor bands in between were observed in ZR75-1, MCF-7, LNCaP and PC3 cells, whereas no detectable amplification product was found in the oestrogen non-responsive MDA-MB231 breast cancer cell line. The first band corresponds well to the expected length (nucleotides 937–1596 of the

oestrogen receptor sequence) of a normal oestrogen receptor mRNA, as determined by comparison with the *ϕx-Hae* III marker fragments. The second band originates from a variant oestrogen receptor mRNA, lacking the entire exon 4; this variant, which is likely to represent a product of alternative splicing, has been isolated and characterized in our laboratories from human breast tumour cell lines (Pfeffer et al 1993). The bands of intermediate length are of unknown origin, although one amplification product shows a length compatible with a putative variant mRNA lacking exon 5, which would also be amplified by the primer set used. Using a nested primer set with an anti-sense primer in exon 4, we observed a single band in all cases; moreover, digestion of the reaction products by the restriction enzyme *Hin*d III, which makes one cut in the amplified region (oestrogen receptor exon 4), yielded identical fragments of the expected size (not shown).

For the first time, we have obtained a normal oestrogen receptor mRNA in LNCaP cells using the RT-PCR system. In addition, a rather abundant expression of a variant oestrogen receptor mRNA is also reported; this variant is jointly expressed with the normal messenger only in oestrogen receptor-positive, oestrogen-responsive MCF-7 and ZR75-1 mammary carcinoma cell lines, whereas both mRNAs are absent from oestrogen receptor-negative, non-responsive MDA-MB231 cells. It is worth mentioning that the amplification primers used (24-mers) show nine and 10 mismatches to the androgen receptor sequence and, if they were to give rise to any amplification product from that sequence, it would not be of the observed length.

Although RT-PCR is not a quantitative technique, the relative amounts of the oestrogen receptor mRNAs were reproducibly different in the cell lines studied (Fig. 3). The levels of expression of both oestrogen receptor mRNAs that were observed in the androgen-sensitive LNCaP cells were similar to those in the MCF-7 and ZR75-1 cells, whereas both mRNAs appeared to be expressed at far lower levels in the androgen non-responsive PC3 cells. The two mammary carcinoma cell lines showed equivalent expression levels, although this was slightly higher in MCF-7 than in ZR75-1 cells. Although the RNA isolated from the PC3 cell line gave rise to low levels of amplification products, no reaction could be observed in the oestrogen non-responsive human breast cancer cell line MDA-MB231. Therefore, the low expression of oestrogen receptor mRNA in PC3 cells is clearly significant and may also account for the apparent lack of soluble oestrogen receptor seen in this cell line in the radioligand binding assay and the absence of cytochemical staining for progesterone receptors.

The oestrogen receptor-associated 27 kDa heat shock protein

As with the larger heat shock proteins (Hsps), a group of small proteins, in the range of 24–30 kDa, have repeatedly been reported to be involved in cellular thermotolerance and response to a miscellany of both growth and

differentiation factors. In particular, recent studies have revealed that the 28 kDa protein found in MCF-7 human mammary cancer cells corresponds to both the oestrogen-related 24 kDa protein and the mammalian Hsp27 (Faucher et al 1993). Interestingly, immunological evidence has indicated that the oestrogen-regulated Hsp27 and the oestrogen receptor-associated 29 kDa protein (p29) are the same molecule (Ciocca & Luque 1991). The p29 phosphoprotein was previously characterized as a cytosolic component of both breast and endometrial cancer tissues that is quantitatively and qualitatively related to the oestrogen receptor but not to other steroid receptors or other binding proteins (King et al 1987). It was proposed that p29 represents a non-hormone-binding component of the receptor mechanism which forms a complex with the oestrogen receptor under certain conditions, such as treatment with ammonium sulphate (Cano et al 1986), and that it may be used as a marker of oestrogen sensitivity in breast and endometrial epithelial cells (King et al 1987) and in cultured fetal Leydig cells (Tsai-Morris et al 1986).

Although several lines of evidence favour the idea that Hsp27 is involved in oestrogen action, it is still uncertain whether this protein actually participates in the processes of association and dissociation of oestrogen receptor heterocomplexes or, alternatively, whether it is involved in the receptor activation and DNA-binding phenomena.

On this basis, we have also inspected expression of Hsp27 in human prostate cancer cells. We immunostained the cells with a D5 monoclonal antibody (Coffer et al 1985) raised against Hsp27 which was visualized through a fluorescein isothiocyanate-conjugated secondary antiserum. Positive staining was observed in both LNCaP and PC3 cells. In LNCaP cells, the majority (over 85%) of cells showed a consistently intense staining, mostly located in the cytoplasm (Fig. 4A). Conversely, PC3 cells displayed a positive staining that was mainly nuclear, although some cytoplasmic staining was also seen (Fig. 4B); the percentage of positive cells was lower than that seen in LNCaP cells (approximately 50%), with minor discrepancies in staining intensity. Control wells from either cell line not receiving the primary antibody stained very poorly, indicating that the Hsp27 staining was specific.

Although a linear correlation between oestrogen receptor content and Hsp27 levels has been reported in human breast tumours, tissues with low levels of oestrogen receptor also exhibited a variable degree of Hsp27 staining (Cano et al 1986). Previous evidence from our laboratories indicated that Hsp27 is associated with nuclear oestrogen receptor in smooth muscle cells of human aorta (Campisi et al 1993). In addition, although the relationship between oestrogen receptor and Hsp27 has been established within individual target tissues, this cannot be true for certain other tissues, including prostate (King et al 1987). Recent studies revealed that presence of Hsp27 is associated with oestrogen receptor in human prostate tumour tissues and is related to the subsequent prognosis for the patients (Ohishi et al 1992).

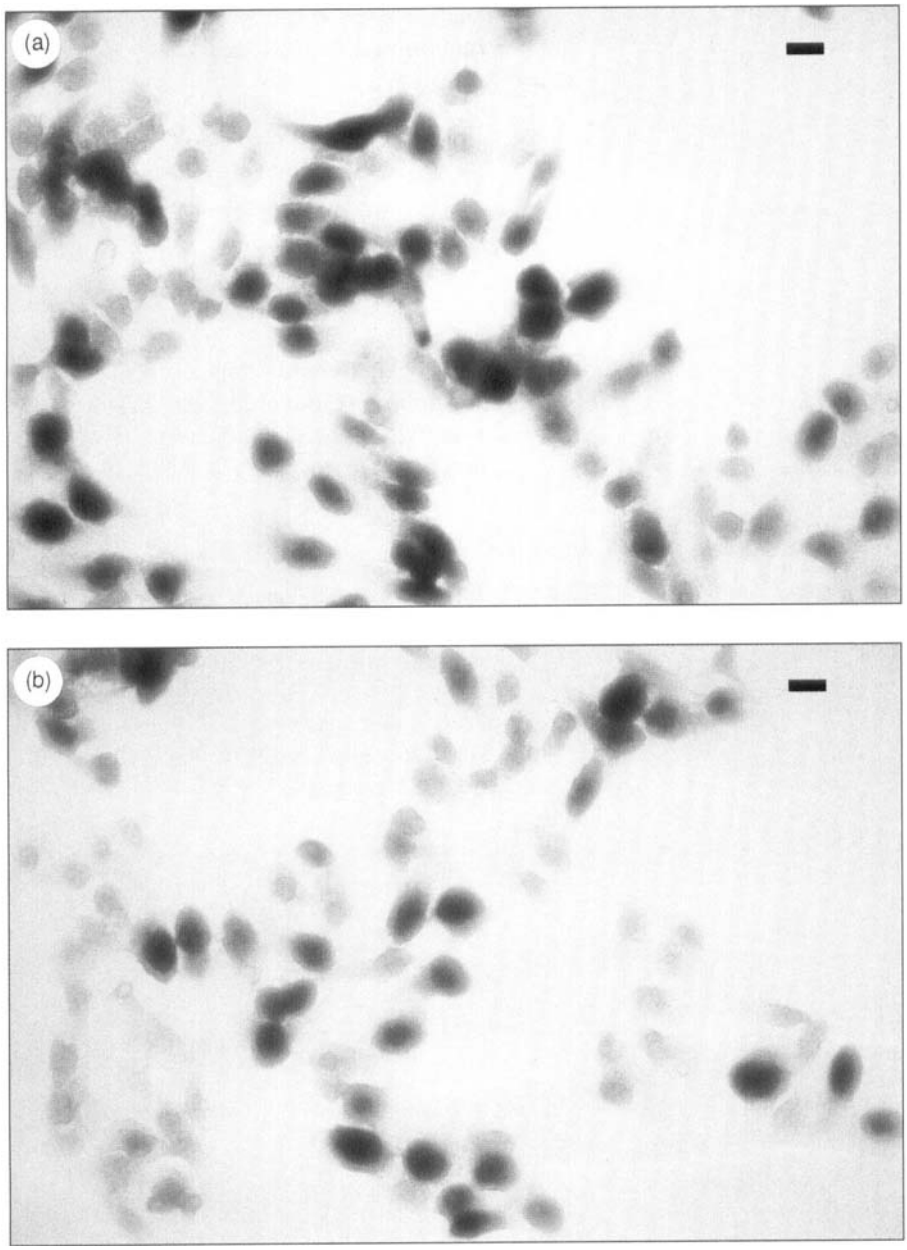

PLATE 2 Immunocytochemistry of oestrogen (a) and progesterone (b) receptors in LNCaP prostate cancer cells. Immunocytochemical assays of both receptors were carried out using modified versions of the oestrogen and progesterone receptor Abbott kits. Cells were incubated for 24 h at 4 °C with primary monoclonal anti-oestrogen receptor or monoclonal anti-progesterone receptor antibodies. The reaction was visualized by means of a peroxidase–anti-peroxidase (PAP) system using diaminobenzydine as the chromogen substrate (bar = 150 μm).

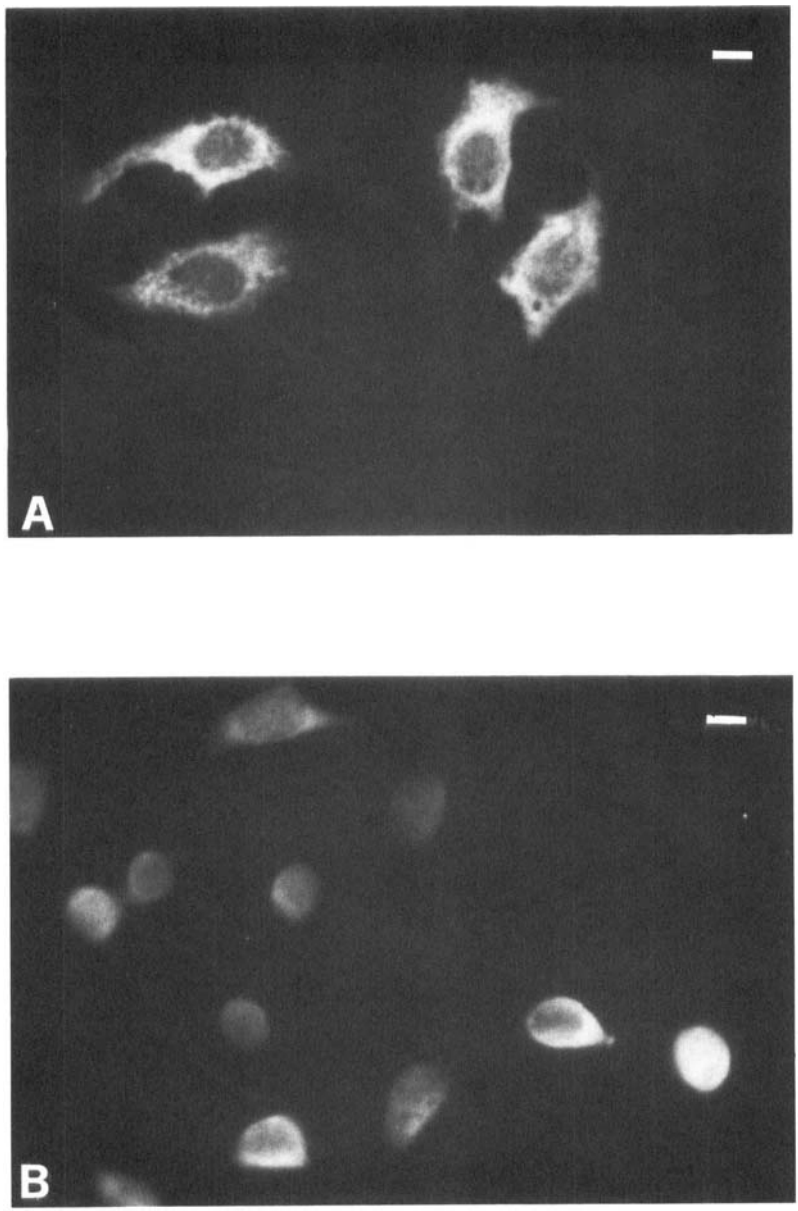

FIG. 4. Immunofluorescence of the 27 kDa heat shock protein (Hsp27) in (A) LNCaP and (B) PC3 prostate cancer cells. Expression of Hsp27 was visualized using a D5 primary monoclonal antibody and a secondary fluorescein isothiocyanate-conjugated antiserum (bar = 150 μm).

TABLE 3 Reports on the oestradiol-induced growth stimulation of LNCaP cells

Author	*Year*	*Stimulation (-fold)*	*Concentration (nM)*
Schuurmans et al	1988	3.5	10
Sonnenschein et al	1989	3.3	10
Veldscholte et al	1991	3.0	10
Kirschenbaum et al	1993	2.0	1–100
Castagnetta et al	1994	2.2	0.01–1

Growth response to oestradiol

Oestradiol-induced growth stimulation of LNCaP cells

Previous studies have consistently reported that oestradiol significantly stimulates proliferation of LNCaP cells (see Table 3) at levels comparable to or even greater than those produced by androgens (Schuurmans et al 1988, Sonnenschein et al 1989, Iguchi et al 1990, Castagnetta et al 1994). It has been proposed that the mitogenic effects of oestradiol on LNCaP cells may be mediated via the point-mutated form of the androgen receptor (Veldscholte et al 1990b). However, the very low RBA values of oestradiol for the androgen receptor (see Table 1) cannot explain its remarkable proliferative potency. Similarly, both progesterone and pregnenolone have been reported to be more potent inducers of growth of LNCaP cells than expected on the basis of their RBA values (Sonnenschein et al 1989). Therefore, the assumption that oestradiol acts via an abnormal androgen receptor appears to be unfounded. We have measured [^{3}H]thymidine incorporation in LNCaP cells cultured for a week in steroid-deprived medium and then exposed for six days to different concentrations of oestradiol (0.01–100 nM), in the presence or absence of the pure anti-oestrogen ICI 182 780 (Wakeling et al 1991). As reported in Table 4, growth of LNCaP cells was significantly increased by all doses of oestradiol. A peak stimulation of over 120% above control values was seen at the 0.01 nM dose ($P < 10^{-6}$). This is not surprising, at least in the present experimental conditions. The simultaneous addition of 100 nM ICI 182 780 completely abolished the oestradiol-induced growth stimulation at any oestradiol concentration, whereas ICI 182 780 itself did not significantly affect cell proliferation. This evidence strongly supports the view that oestradiol acts via its own receptor. This finding is also in accord with a previous observation that the pure anti-oestrogen EM 139 not only reversed the androgen-induced increase in LNCaP proliferation, but also significantly inhibited basal cell growth (de Launoit et al 1991).

TABLE 4 Effects of oestradiol and ICI 182 780 on tritiated thymidine uptake by LNCaP cells

Treatment	*[^{3}H]thymidine uptake (dpm)*
None	38 538 ± 1799
0.01 nM oestradiol	87 255 ± 9459
1 nM oestradiol	64 766 ± 5297
100 nM oestradiol	54 789 ± 2928
ICI 182 780[a]	37 267 ± 1933
ICI 182 780[a] + 0.01 nM oestradiol	36 932 ± 1760
ICI 182 780[a] + 1 nM oestradiol	36 832 ± 1706
ICI 182 780[a] + 100 nM oestradiol	37 586 ± 1202

[a]100 nM.
Values represent mean disintegrations per minute (dpm) ± SD; $n = 4$. Cells, grown in steroid-deprived medium, were exposed to increasing concentrations of oestradiol, with or without ICI 182 780, or to ICI 182 780 alone. Cell proliferation was measured by means of a six-hour pulse of [^{3}H]thymidine and an estimate of the acid-precipitable counts.

Oestradiol-induced growth inhibition of PC3 cells

Further interesting evidence comes from the androgen-unresponsive PC3 cells. As illustrated in Fig. 5, oestradiol exerts a clear inhibitory activity on growth of these cells. As can be seen, after six days' exposure, PC3 cells displayed a dose-related inhibition of growth, which was maximal at 10^{-7} M oestradiol (55.2% with respect to control; $P < 10^{-6}$) but still significant at physiological concentrations. At higher doses of oestradiol (1 nM or more) this effect became evident after 72 h incubation (not shown). This negative growth regulation is cognate to that observed in PC3 cells after the addition of 1 ng/ml transforming growth factor (TGF)-β1 (54.4% of control) under exactly the same experimental conditions. Consequently, we have explored the possibility that the oestradiol-induced growth inhibition of PC3 cells is mediated by TGF-β. Interestingly, the addition of a neutralizing anti-TGF-β1 antibody in stringent experimental conditions provoked a remarkable increase (close to 300% of control) in cell proliferation (Fig. 5), suggesting the presence of high levels of endogenous TGF-β1 in PC3 cells. This effect was opposed by the addition of oestradiol (10^{-11} to 10^{-7} M), and was almost completely reversed at the 100 nM dose (Fig. 5). However, data from Northern blot analysis indicate that different oestradiol doses (10^{-11} to 10^{-7} M) do not affect TGF-β1 mRNA expression in PC3 cells after various (six, 24 or 72 h) incubation times (Carruba et al 1994a). This is not unexpected, since Knabbe et al (1987) found that anti-oestrogens such as tamoxifen have no influence on TGF-β1 mRNA in the oestrogen-dependent MCF-7 human breast cancer cells, although tamoxifen

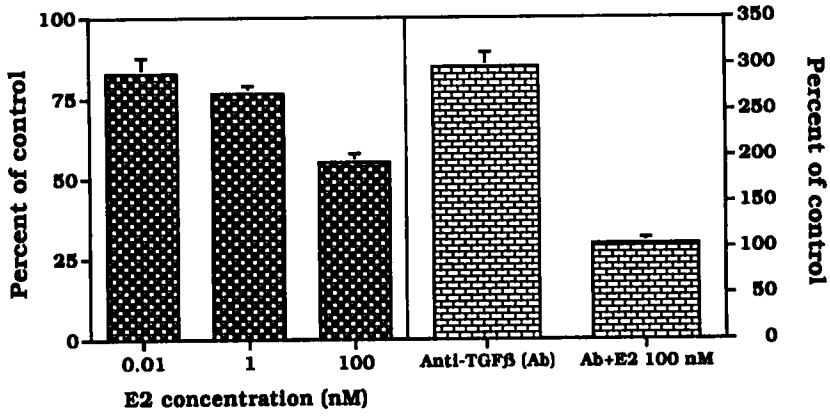

FIG. 5. Growth effects of oestradiol and anti-transforming growth factor (TGF)-β1 antibody in PC3 cells. Cells were exposed for six days to increasing concentrations (0.01 to 100 nM) of 17β-oestradiol (E_2) or anti-TGF-β1 (Ab) antibody (9.2 μg/ml) ± 100 nM E_2. Values represent means ± SEM (bars) of $n = 3$ experiments. Degrees of significance (P) with respect to controls (two-tailed Student's t-test, 95% confidence limits): 0.01 nM E_2, $P < 0.0002$; 1 nM, $P < 0.00002$; 100 nM, $P < 10^{-6}$; Ab, $P < 0.00001$; Ab + 100 nM E_2, $P = 0.435$ (no significant difference).

treatment increased TGF-β1 production (up to 27-fold) in this cell line. More recent studies have revealed that levels of TGF-β1 mRNA in either oestrogen receptor-positive breast cancer cell lines (MCF-7 or ZR75-1), or oestrogen receptor-negative cancer cells (such as mammary MDA-MB231 [Arrick et al 1990] and endometrial HEC-50 [Anzai et al 1992]) remain unchanged following oestradiol administration. It is noteworthy that the very same oestrogen receptor-negative human cancer cells have been reported to be exquisitely sensitive to TGF-β1 (Arteaga et al 1988). Equally, growth of PC3 cells has been reported to be remarkably affected by TGF-β1, contrary to what occurs in LNCaP cells (Wilding et al 1989, Carruba et al 1994b).

Perspectives

Previous evidence from either *in vivo* or *in vitro* studies clearly suggests that oestrogens may play a role in the regulation of prostate cancer cell growth. In addition, the presence of high-affinity binding proteins for oestradiol has been revealed in both hyperplastic and cancerous human prostate tissues (Murphy et al 1980, Donnelly et al 1983, Ekman et al 1983). The results presented here show that oestradiol negatively regulates the growth of PC3 cells, but in LNCaP cells it behaves as a mitogen at physiological (10^{-11} to 10^{-9} M) concentrations; in addition, exposure of LNCaP cells to higher doses of oestradiol may produce, in stringent experimental conditions, a decrease in cell

proliferation (L. Castagnetta et al, unpublished work). This biphasic response of LNCaP cells to oestradiol parallels that repeatedly observed for androgens by different research groups, including our own (Sonnenschein et al 1989, Carruba et al 1994b).

The oestradiol-induced growth stimulation of LNCaP cells appears to be mediated via specific, high-affinity binding proteins that are refractory to any interfering androgen, such as R1881. In addition, the consistent expression of both the progesterone receptor and Hsp27 supports the presence of an intact action mechanism of oestrogen. This view is also corroborated by evidence that the addition of the pure anti-oestrogen ICI 182 780 entirely abolishes the mitogenic effect of oestradiol on LNCaP cells. The latter evidence, coupled with the inconsistency between RBA values and relative proliferative potency of oestradiol, leads us to conclude that the effects of oestradiol on the growth of LNCaP cells are totally independent of the point-mutated form of the androgen receptor. Incidentally, type I and II oestrogen receptor content is remarkably (over fivefold) higher than that of the androgen receptor we have recently measured in LNCaP cells (Carruba et al 1994b).

The overall functions of oestrogen in human prostate cancer, at least in these *in vitro* systems, appear to be more complex and disparate than expected. On the one hand, mechanisms responsible for the oestradiol-induced growth inhibition of PC3 cells may help explain the beneficial effects of pharmacological doses of oestrogen in advanced human prostate cancer, even in the androgen-independent state. On the other hand, the evidence that oestradiol stimulates growth of LNCaP cells may account for results previously obtained in animal model systems, favouring the resulting concept of an androgen-supported oestrogen-enhanced stimulation of prostate epithelium as an essential requirement for overcoming factors that normally limit cell proliferation (Leav et al 1989). The question of whether the different oestrogen receptor expression levels observed in LNCaP and PC3 cells may somehow be related to their distinct biological response to oestradiol is worthy of further investigation.

Overall, the varied activities triggered by oestradiol strongly suggest, at least *in vitro*, a direct role for oestrogen function in the regulation of the growth of prostate cancer cells. Consequently, the current criterion of hormone-dependence of human prostatic cancer should be reassessed.

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DISCUSSION

Baulieu: I was surprised at the relatively low numbers of prostatic cancers in the USA. I thought that almost all men over the age of 80 developed prostatic cancer. You quoted a figure of about 200 000.

Castagnetta: That was 200 000 new cases per year of clinically manifest human prostatic carcinoma, i.e. twice the number expected in 1990 (Garnick 1994).

Robel: Have you found significant amounts of oestrogen receptor in the tumours of your patients?

Castagnetta: Yes, we have clear evidence for both type I (high affinity, low capacity) and type II (lower affinity, greater capacity) sites of oestrogen binding in benign and malignant human prostate, although receptor content and distribution vary considerably in different tissues.

Baulieu: You haven't considered that, as well as epithelial cells, the prostate is made up of mesenchymal cells, and that these cells may or may not have the oestrogen receptor. You haven't considered the development of the tumour in the prostate in terms of other cells responding to all sorts of signals, including steroids.

Castagnetta: That is an interesting point, but it is mostly outside the aims of this study. We have evidence that oestrogen actively stimulates growth of LNCaP cells via an oestrogen receptor-mediated mechanism (Castagnetta et al 1994); additionally, oestrogen induces a dose-dependent inhibition of the proliferative activity of the androgen non-responsive PC3 cells (Carruba et al 1994). This would imply that oestrogen may be effective in the treatment of advanced, hormone refractory disease. On the other hand, the oestrogen-induced stimulation of growth may also be counteracted by progesterone via a progesterone receptor-mediated mechanism. This is another indication as to how prostate cancer cells can be regulated.

Baulieu: Some people have used progestogens to treat prostatic cancer. Can you explain the scientific basis for this sort of therapy?

Castagnetta: Not from this study. However, there is evidence that progesterone down-modulates oestrogen receptors in human breast cancer cells.

Oelkers: It could suppress luteinizing hormone instead of gonadal functions.

Baulieu: But this is progesterone administered locally, not orally.

Jensen: You have used two cell lines, PC3 and LNCaP. Which do you consider to be most representative of human prostatic cancers? Do either of these cell lines synthesize prostate-specific antigen (PSA)?

Castagnetta: Maybe both, if we consider that human prostate cancer is commonly composed of both androgen-dependent and androgen-insensitive cell clones. We have found that expression of PSA is very low in LNCaP cells unless they are stimulated with androgens or dihydroxyvitamin D_3 (Skowronski et al 1993). In contrast, PC3 cells do not stain for PSA under any conditions.

Jensen: Then, in answer to my question, the LNCaP cells seem to be more like the human cancers, especially the androgen-dependent ones.

Castagnetta: I agree that the LNCaP cells are much closer to those of a steroid-responsive prostatic carcinoma. However, we have evidence that primary cultures of prostate tumour cells with high levels of PSA may rapidly become PSA negative when they are cultured.

Rochefort: If you think that oestrogen stimulates the growth of prostate cancer, would you assume that anti-oestrogen therapy would be as effective as or more effective than anti-androgen therapy in prostate cancer?

Castagnetta: This study suggests that the combination of anti-oestrogens and anti-androgens could provide additional benefit in the management of at least a subset of prostate cancer patients. Evidently, one should place very wide confidence intervals around the extrapolation of data from the *in vitro* to the *in vivo* condition, and vice versa.

Rochefort: We have to explain the *in vivo* situation first.

Castagnetta: Of course. I'm hoping we will get some answers in the next two years from clinical trials using anti-oestrogen therapy, alone or in combination, as first-line treatment for prostate cancer patients. The idea is to embark on a multicentre study in the framework of the Italian Task Force on Human Prostate Cancer (PONCAP).

Baulieu: And along these lines, have you measured circulating oestrogens in prostatic cancer?

Castagnetta: No. We thought it more appropriate to look at tissue content rather than plasma levels of relevant steroids.

Manolagas: Why do prostate cancer cells metastasize so readily to bone?

Castagnetta: Bone is a target tissue for oestrogens. One could speculate that treatment of advanced prostate cancer metastatic to bone with anti-oestrogens would be beneficial; anti-oestrogens such as tamoxifen, have been reported to stimulate TGF-β production in human breast tumour cells (Knabbe et al 1987).

Manolagas: The only suggestion I had is that bone is loaded with TGF-β. If prostate cancer is so sensitive to and dependent on TGF-β, this might be one possibility.

Castagnetta: It ought to be emphasized that oestrogens regulate TGF-β synthesis in bone cells, such as osteoblasts.

Bonewald: The interesting thing about prostate cancer is that not only does it make TGF-β, but it apparently also makes a latent form that lacks the TGF-β-binding protein (Eklöv et al 1993). This may play a role in what you're seeing—you may have an activation process going on and that's why you're seeing a TGF-β response.

More importantly, bone morphogenetic proteins are produced by prostate cancers (Harris et al 1994). These proteins are also expressed in normal prostate tissues. It may be that in the cancerous state there is over-expression of certain forms of the bone morphogenetic proteins, which predisposes this tissue to bone.

Jensen: It has long been known that prostatic cancers usually proliferate rather slowly, especially in the older patient, but once metastases are established in bone, they grow very rapidly. Recently, it has been shown that transferrin, produced in the bone marrow, acts as a stimulatory factor for the growth of prostate cancer cells (Rossi & Zetter 1992).

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Final discussion

Beato: I wanted to comment on the last three talks and, in particular, on the mechanisms by which cells become hormone resistant.

My impression has been that there are two groups of findings. In many cases resistance is related to the receptors—for instance, progesterone receptor variants that may change the behaviour of the cells. But in other cases resistance may arise without receptors being affected, for instance because different family members of the AP-1 complex are found in different cells, which then respond in a different way. This a very interesting possibility that could be discussed.

Horwitz: All hormone-dependent cancers, and that includes breast and prostate cancers, become resistant to endocrine treatments after a period of positive response. Stage IV breast cancers are destined to become resistant to hormone treatments, which commonly means that they become resistant to tamoxifen. Why does this occur? In the past, the simple explanation was that tumours lose hormone receptors: hence, they lose their ability to respond to the anti-oestrogen. However, this explanation is untenable, since 50% of hormone-resistant breast cancers retain oestrogen receptors. An alternative explanation, which is relevant to steroid antagonists like tamoxifen and the antiprogestins that I have been discussing, is that tumour cells change. As a tumour progresses, new protein factors are expressed and new genes are activated. If these factors are transcriptional coactivators for steroid receptors, or if the genes are receptor regulated, then new responses to the antagonist can be expected. Thus, the anti-oestrogen or antiprogestin may be perceived as an agonist, not an antagonist in this advanced tumour. One can imagine that there would be selection pressure in favour of this progression—cells that respond to the antihormone as an inhibitor would be suppressed, whereas cells that respond to the antihormone as an agonist would proliferate. The latter cells would be classified as 'hormone resistant', when in fact they remain quite sensitive to the hormone. Thus, the hormone has not changed and the receptor has not changed: it is the cancer cells that have changed. Parenthetically, this is referred to in the clinical literature as a 'tamoxifen withdrawal response'. Patients whose tumours are initially inhibited by tamoxifen, but then resume proliferation while they are still receiving tamoxifen, often experience a remission when tamoxifen is withdrawn. Thus, tamoxifen has switched from being an antagonist to being an agonist. This switch can occur without invoking changes in the receptors or the ligands. On the other hand, in some tumours, mutations in the receptors could account for functional switching, and this too has been described (Horwitz 1994).

Baulieu: How are we going to transfer this sort information to the clinic? It is a very difficult task. I remember that at the beginning of the RU486 story it seemed that it would be very simple to use this antiprogestin for pregnancy interruption. Soon after, we published that it was also an agonist (Gravanis et al 1985). Interestingly, the people at Roussel were furious that we published such a 'complexity', fearing it would destroy the image of the compound. We had some conflict with those who wanted to simplify the story and eventually mislead the public. After all, our final goal is to help people, not to praise the so-called wonder properties of a drug.

Manolagas: A huge problem in clinical medicine is the resistance of multiple myeloma to glucocorticoids. Practically every multiple myeloma will respond very well to glucocorticoid steroids for the first six to eight months, and then most of them lose responsiveness. Several leukaemias do the same thing. This concept and a lot of the discussions that have taken place over the past three days have led to a realization that we cannot work in vacuum. We cannot just take a gene, a receptor and a ligand and try to explain the whole story; we should be aware of interactions within the cell, as well as outside the cell. There is an integration of signals coming from numerous transcription factors within the cell and, as our data now suggest, also from outside the cell. The immune, endocrine and neural systems 'talk' all the time. Rather than being abstractionist, we have to be more constructionist and consider the whole organism. We have to try to go as fast as we can from the *in vitro* to the *in vivo*, from the animal to the human.

Baulieu: This has been demonstrated for several peptides, such as somatostatin (which is produced in the brain and pancreas) and now also for steroids produced in the brain. It seems that many molecules are (1) produced at different sites from the same genome, and not only in some cells originally discovered as synthesizing cells, and (2) more and more we have local systems of distribution. These are extremely difficult to work with because we don't know how to explore them in an integrated organism. I think we are in a sort of crisis; there are too many properties already known which we cannot apply for sure to what we are interested in *in vivo*, and particularly in pathological states.

Beato: I want to add a word of caution. In the glucocorticoid field, we have been biased by the original findings on selection of lymphoid cells resistant to killing by glucocorticoids (Gehring 1986). Ninety-nine per cent of all the resistant cells had receptor mutants: either they were receptor deficient, or they had receptors that didn't move to the nucleus properly, or some other defect. It was virtually impossible at that time to find other partners involved in the cytostatic reaction. The impression was that the main player in the action of glucocorticoids on the lymphoid system was the receptor. And yet we recently looked at chronic lymphatic leukaemia patients who have become resistant to glucocorticoid therapy. We carried out polymerase chain reaction sequencing of the receptor, and there was not a single case of mutation of the receptor (Soufi et al 1994).

Manolagas: Are you surprised that systemic hormones, which come from a distance, require local control to modify their actions? If you consider that a hormone coming from a gland can affect many different tissues, you will see that its action needs to be modified locally to accommodate local needs. So the cross-talk between systemic and local signals is something we should have been expecting.

Baulieu: One aspect we often neglect is that of the access of signalling molecules to tissues. It is not just a matter of diffusion, but also of permeability of vessels, binding of protein, or lipid accumulation. Another aspect is the effects of continuous versus pulsatile hormone treatment.

McEwen: There is an example of pulsatile versus continuous oestrogen treatment and the activation of sexual behaviour: it has been claimed that intermittent application of oestradiol to male rats activates sexual behaviour whereas continuous treatment doesn't (Södersten et al 1989, Moreines et al 1986).

Baulieu: That's very difficult to understand *in vitro*, because we have to take into account not only the receptor and its regulation, but also the half-lives of all the biological consequences of its activation.

Oelkers: Another exciting new mechanism involving local factors that regulate access of steroids to their receptors is the role of 11-hydroxysteroid dehydrogenase (11-HSD). The human aldosterone receptor has been recently cloned and it was found that it cannot distinguish between hydrocortisone and aldosterone. However, the receptor is governed by aldosterone, although concentrations of free cortisol in plasma are 100 times higher. Then it was found that the collecting duct cells of the kidney that harbour the receptor are very rich in the enzyme 11-HSD that converts cortisol to cortisone, which cannot bind to the receptor. Aldosterone is not a substrate of this enzyme. Thus, substrate or steroid specificity is conferred to the cell by 11-HSD. This mechanism is also important for the type I corticosteroid receptor in the brain.

Bonewald: With reference to Miguel Beato's comment about being misled by the individual cell systems that we're looking at, often when we want to address a problem we find a window and that's where we look. Perhaps we're not looking through the right window. Maybe we need to be looking at the normal processes: we need to be looking at the primary cell, and how oestrogen functions in the normal immune response or on the normal mammary cell first, and then extrapolate from the normal to the perturbed system.

Baulieu: It isn't very easy to do quantitative work with normal cells *in vitro*, because primary cultures often give different results when we repeat the experiments.

Bonewald: A prime example is the prostate cell lines. We have looked at three prostate cell lines and we get totally different profiles when we look at factors that induce bone formation, for example. For this reason we are trying

to go back to the normal prostate tissue, but it's hard to find normal prostate to work with.

Rochefort: Concerning *in vitro* versus *in vivo* results, recent reports have suggested that the shape of cells is important. The results we can get from growing cells on plastic may not be totally relevant to what happens *in vivo*. Cells grown on extracellular matrix-like matrigel, for instance, behave differently from those grown on plastic culture plates.

Baulieu: The anti-oestrogen story is very clear—anti-oestrogen activity with no agonistic activity is easily obtained in many cells *in vitro*, whereas the corresponding cells *in vivo* may undergo some agonistic response.

Rochefort: The different regulation of cathepsin D by the anti-oestrogen tamoxifen *in vivo* versus *in vitro* may be explained at the post-translational level rather than at the transcriptional level. More generally, breast cancer cell lines like to grow on plastic, since they have been selected for this ability and cell polarity may not be important. Normal mammary cells grown on plastic lose their polarity and cannot organize themselves into a glandular structure as in a three-dimensional matrix or as *in vivo*. There is an interesting molecule called calreticulin, which has a consensus amino acid sequence which recognizes both the intracellular domain of the integrin α-subunit at the plasma membrane level, and the region between the two zinc fingers of nuclear steroid receptors (Dehar et al 1994, Burns et al 1994). This molecule may provide an explanation of how cells can respond differently at the gene level depending on whether they are growing on plastic or growing in a three-dimensional extracellular matrix.

Horwitz: With regard to model systems, I think you have all heard the analogy of the three blind men, each trying to describe the same elephant. One feels the tusk and concludes that the elephant is smooth and has a sharp point. Another feels the trunk and concludes that the elephant is wrinkled. The third feels the tail and concludes that it is skinny and hairy. None of them, of course, sees the big picture. In a similar way, we're all looking at parts of the elephant, and some day we hope to be able to integrate all of our information and come up with an accurate picture of the whole elephant. None of us, in our microsphere of activity, can come up with the entire story—we have to listen to one another if we are to assemble the whole from the parts.

Baulieu: This meeting has been rather good in this respect, because people have presented their results in terms which are comprehensible to scientists from diverse backgrounds. We have been concerned with both basic and medically applicable concepts. Certainly, there is still a long way to go, but I think we should avoid being too sceptical about the results already obtained.

One issue that we haven't tackled is how we should respond to proposals to give anti-hormones prophylactically to healthy people 'at risk' of breast cancer. From from what we know, if we take literally what we have discussed here, we shouldn't do anything. On the other hand, it can be argued that we don't have

the right to do nothing. Rather pompously, people like to call these issues 'ethics'. I don't know who is going to discuss this and make a decision, but clearly it takes courage. People often don't realize that scientists are not computers, but we have to make difficult 'human' decisions. Nobody doubts that the background for making these decisions is the sort of results that have been reported here. It is not enough, but it is necessary.

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