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THE ROLE OF RECEPTORS IN THE STEROIDAL REGULATION OF TUMOUR CELL PROLIFERATION

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SUMMARY

The relevance of steroid-receptor content of mammary tumour cells to the growth promoting effect of steroids is discussed by reference to the effects of androgens and oestrogens on growth of mouse mammary tumour cells (S115 cells) in cell culture. Proliferation of these cells is increased by physiological levels of androgen and evidence is presented that the response is related to the presence of androgen receptors in S115 cells. The S115 cells also contain an oestradiol receptor that is unrelated to proliferative responses. Thus one cannot automatically equate receptor content to proliferation rate.

INTRODUCTION

The interaction of hormonal steroids with specific receptor proteins [R] is now recognized to be the initial step in most actions of these hormones. A clinically useful test for hormone responsiveness of human mammary tumours is based on the supposition that responsive tumours should contain specific receptors for the effective steroid whilst unresponsive tumours would be receptor negative. The clinical data obtained with human breast tumours clearly indicate that the majority of R_E-negative tumours are indeed unresponsive to a spectrum of hormonal therapies but that 40–50% of the R_E-positive tumours are also unresponsive [1–2]. Many explanations have been advanced for the occurrence of these so-called 'false-positive' tumours, the most important of which are listed in Table 1. Given the heterogeneous nature of breast cancer it is probable that no one of the factors listed in Table 1 provides the complete answer. Detailed discussion of most of the points listed in Table 1 have been published [3, 4]. The present communication will concentrate on two of the points listed in Table 1, namely the modulating effect of steroids and the question of whether all steroid receptors found in mammary tumours are related to proliferative responses. Data obtained from a mouse mammary tumour cell line (S115 cells) in culture will be used to illustrate the points we wish to make.

Table 1. Potential reasons for 'false positive' tumours

1. Mixed cell types
2. Absence of activation/nuclear transfer of receptor
3. Steroids are only 'modulating agents'?
4. Number of receptors required for a response?
5. Assumption that presence of receptor is related to proliferative response?
6. Does receptor content for one hormone indicate response to all hormone therapies?
7. Clinical assessment

S115 mouse mammary tumour

The original tumour arose spontaneously in a female mouse and grew equally well when transplanted into male and female hosts; after 18 passages it would only grow in male mice and had clearly become androgen-responsive [5]. Cells derived from this tumour retain their androgen responsiveness in cell culture and the characteristics of such cultures have been reviewed elsewhere [4]. Two properties are important in relation to the present communication. First, the cells grow well in the absence of androgen; addition of either testosterone (T) or 5α-dihydrotestosterone (DHT) to the culture medium only increases the rate of proliferation two to threefold. Secondly, as reported by Jung-Testas, Desmond and Baulieu [6] the S115 cells contain two distinct receptor sites with different ligand specificities, an androgen binding site (R_A) and an oestrogen binding site (R_E).

Modulating effect of steroids

It is now fairly clear that steroids affect cell proliferation by amplifying an existing series of events rather than switching on new processes. This is evident from all the experiments on steroid stimulation of proliferation in culture [3] and, for this reason, we prefer to use the term steroid 'responsive' rather than steroid 'dependent'. The behaviour of S115 cells in culture conform to this view but interestingly, tumours growing in mice exhibit a greater sensitivity to androgen than do cells in culture. The explanation for this discrepancy seems to reside in the simpler pattern of growth control in culture as compared with the whole animal. Under our conditions of culture, the three main factors regulating proliferation are serum concentration, cell density and androgen concentration. In the animal, additional constraints exist such as immunological response of the host, the presence of multiple hormones, a much more complex interaction

Table 2. Comparison of cytosol receptor affinities and biological effectiveness of sex hormones

Hormone	Binding K_D ($\times 10^{10}$ M)	Binding n (F mol/mg protein)	Biological activity Conc. (M $\times 10^{10}$) for half-maximal effect: Stimulation	Inhibition of T effect
Dihydrotestosterone	8 ± 2 (5)	57 ± 11 (5)	10	
Testosterone	1 ± 1 (4)	47 ± 9 (4)	10	
Oestradiol			$\sim 10^4$	$\sim 10^4$

Binding data were obtained by Scatchard analysis [10] after overnight equilibration of cytosol with ^{3}H ligand at 4°C and removal of unbound steroid by treatment with Dextran-charcoal for 10 min at 4°C. Results are mean ± S.E. (No. observations). The biological activity data are taken from Fig. 1.

between cells and a variable blood supply. Furthermore, in the animal, the responsiveness of a tumour is usually assessed by whether hormone treatment changes the size of the tumour; tumour size reflects a balance between cell proliferation and death [3]. In the animal, cell death can be a major factor in determining tumour size but cell death is much less important under our culture conditions. Thus, one could have a tumour made up of receptor-positive, hormone-responsive cells that on hormone therapy would not change its size to any appreciable extent; this would occur if the hormone effect was small relative to the other stimuli impinging on the tumour.

The role of androgen (R_A) and oestrogen (R_E) receptors in determining proliferation

S115 cells contain approximately equal numbers of R_A and R_E sites [6, 7]. R_A recognizes both DHT and T and, to a lesser extent oestradiol; it has no measurable affinity for diethylstilboestrol (DES). R_E on the other hand, has a high affinity for oestradiol, an approximately ten-fold lower affinity for DES and does not recognize DHT or T. The binding constants for DHT, T and oestradiol are shown in Table 2. When [^{3}H]-oestradiol was used as the ligand, no significant differences in binding constants was obtained between experiments in which DHT was or was not added to the cytosol; the values for K_D and n (oestradiol) thus relate to the high affinity R_E sites rather than the weaker R_A sites. We have found it difficult thus far to get an accurate measurement of the affinity of oestradiol for the R_A site, but the seventeen-fold lower affinity of oestradiol relative to DHT reported by Jung-Testas *et al.* [6] is probably a minimal value.

From these data one can make certain predictions about the growth promoting properties of the various sex hormones depending on whether R_A or R_E are involved in the growth response. If R_A but not R_E is important, DHT and T should stimulate proliferation with a half-maximal effect at about 10^{-9} M; oestradiol at high concentration should antagonize the effect of the androgen. Oestradiol in the absence of androgen might either inhibit or weakly stimulate growth. More importantly, DES should not affect androgen-induced proliferation. If R_E is involved in proliferation, oestradiol should be effective at about 10^{-9}–10^{-10} M as should DES.

The data strongly support the view that R_A but not R_E is involved in stimulating proliferation. Both DHT and T stimulate proliferation with a half-maximal effect at approx. 10^{-9} M steroid (Fig. 1 and Table 2); oestradiol is ineffective below 10^{-6} M and is only weakly stimulatory at higher concentrations. DES has no effect on proliferation until very high ($>10^{-6}$ M) levels are used when an inhibition is observed. Oestradiol at $>10^{-7}$ M antagonizes the androgen effect as does DES at even higher concentrations (Fig. 2). It would thus seem that oestradiol acts as a partial agonist *via* R_A but does not affect proliferation at a concentration sufficient to saturate R_E. At concentrations of oestrogen up to and including 10^{-6} M, there is no evidence to necessitate postulating a functional role for R_E in the control of proliferation. The evidence is insufficient to rule out the possibility that

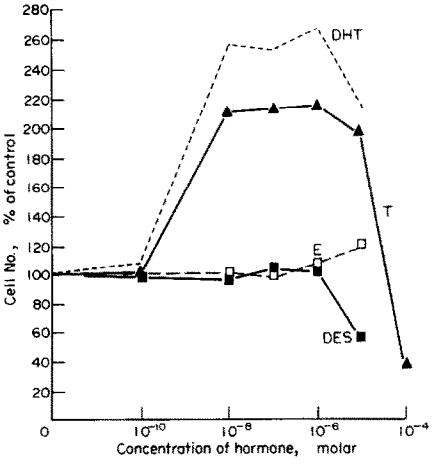

Fig. 1. Effect of sex hormones on growth of S115 cells. Hormones were added to the culture medium 6 h after plating. Medium plus hormones were changed after a further 3 days and cell number measured with a Coulter counter after another 1 day. DHT—5α-dihydrotestosterone; T—testosterone; E—oestradiol; DES—diethylstilboesterol.

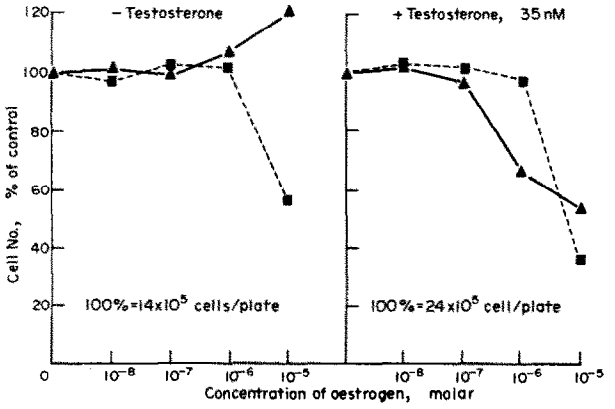

Fig. 2. Effect of diethylstilboestrol (----) and oestradiol (——) on growth of S115 cells in the presence and absence of testosterone. Experimental conditions were as described in the legend of Fig. 1.

R_E is important at 10^{-5} M oestrogen but this is unlikely when one considers the K_D of R_E for oestradiol (Table 2).

Further support for a positive role for R_A comes from experiments with the antiandrogens cyproterone acetate (6α-chloro-17α-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione-17-acetate) and BOMT (6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one). Both these agents compete with DHT for R_A sites. The competition is relatively poor and it has been difficult to get reliable measurements of K_i values which are of the order of 10^{-7} M. BOMT and cyproterone acetate have no significant effect on proliferation on their own (Fig. 3). Cyproterone acetate has an anti-androgenic effect at lower concentrations than does BOMT (Fig. 3).

Long-term culture of S115 cells in the presence and absence of androgen

When S115 cells are cultured in the continued absence of androgen they lose their responsiveness to added androgen [4]. As those experiments were carried out with uncloned cells it could be argued that the loss of responsiveness was due to the selection of unresponsive cells from a mixed population. These experiments have now been repeated using cloned cells with similar results (Fig. 4). Cells grown in the continual presence of androgen retain their responsiveness for the duration of the experiment whereas androgen deprived cells lose their responsiveness after 3–4 weeks culture. A uniform feature of this type of experiment is a concomitant loss of responsiveness and a five-fold reduction in growth

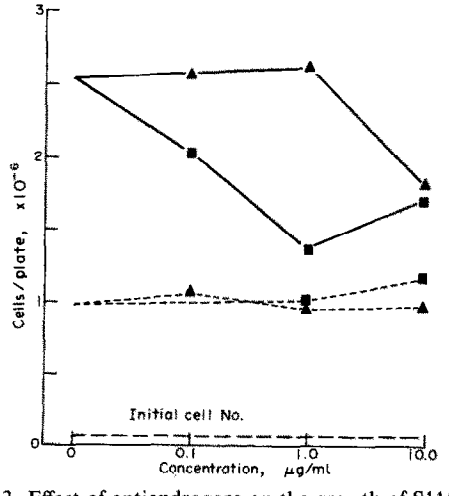

Fig. 3. Effect of antiandrogens on the growth of S115 cells in the presence (——) or absence (----) of testosterone. Experimental conditions were as described in the legend of Fig. 1. Testosterone was added at 0.01 μg/ml medium. ■—cyproterone acetate; ▲—BOMT.

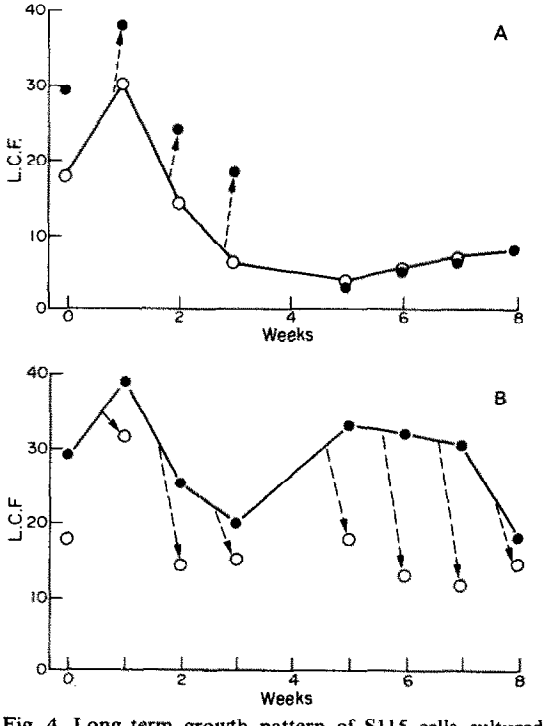

Fig. 4. Long term growth pattern of S115 cells cultured in the absence (A) or presence (B) of testosterone (0.01 μg/ml medium). The experimental design is illustrated in Fig. 5. L.C.F. [^{3}H]-thymidine-labelled cells/microscope field. ●—testosterone added 24 h prior to [^{3}H]-thymidine labelling; ○—testosterone absent.

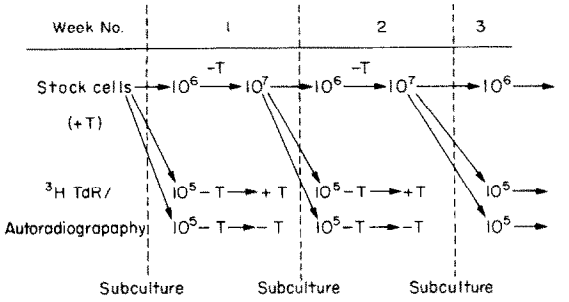

Fig. 5. Experimental design for testing effect of prolonged androgen deprivation on growth pattern of S115 cells. This Fig. illustrates the treatment of testosterone-deprived (-T) cells. A parallel experiment was carried out with testosterone maintained cells. Where indicated, testosterone was added at 0.01 μg/ml medium. The numbers refer to cells/plate. [^{3}H]-TdR, [^{3}H]-thymidine.

rate in the absence of androgen. The design of this experiment as illustrated in Fig. 5 is such that an explanation based on selection of an unresponsive population of cells is not tenable. If a mutation rate of $1:10^6$ cell generations [8, 9] occurs in S115 cells, between 1–3 additional mutant cells would be carried over from one week's experiment to the next. Thus, at no stage of the experiment would the 'normal' cells represent less than 99% of the total cell population. Furthermore, the unresponsive cells grow more slowly than their responsive counterparts which would favour the selection of responsive rather than unresponsive cells. It therefore seems more probable that the rapid loss of responsiveness to androgen, coupled with a general decrease in growth rate, represents a phenotypic rather than genotypic change.

When specific [^{3}H]-DHT binding of S115 cells was measured throughout the experiment just described loss of responsiveness to androgen and fall in growth rate was accompanied by a 50% decrease of specific [^{3}H]-DHT binding from the normal value of about 5000 molecules R_A per diploid genome. It is not possible to say at present if the fall in binding is a cause or effect of the altered growth characteristics. However, other experiments (not shown) indicate that alterations in growth rate *per se* do not alter R_A levels. We thus favour the view that a fall in R_A is a causative factor in changing the growth pattern. The reason why R_A should fall rapidly after 3–4 weeks of androgen deprivation is not known but the most likely explanation is that R_A synthesis is regulated by androgens. It should be noted that the unresponsive cells do contain measurable amounts of R_A. The interesting observation that growth rate in the absence of androgen declines with the fall in R_A also warrants further study. On the basis of the experiments reported here it has been speculated that steroid receptor proteins might be biologically effective in the absence of ligand, their efficiency being increased in the presence of steroid [3].

CONCLUSIONS

These experiments with androgen responsive mammary tumour cells illustrate two ways in which detection of specific receptors is not correlated with proliferative response. (1) The cells contain normal levels of oestrogen receptor whose characteristics suggest that the receptor is not involved in proliferation. (2) Androgen-unresponsive cells can be produced that contain measurable amounts of androgen receptor.

Furthermore, as steroids only have a modulating effect of proliferation, it is suggested that whether hormone treatment does or does not affect tumour size *in vivo* depends on the magnitude of the steroid effect relative to other factors impinging on the tumour.

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DISCUSSION

Rousseau. In view of the known effects of steroids on RNA tumour viruses, do you know whether your cells produce B type particles?

King. No.

Pasqualini. In one of the first pictures you showed maximum stimulation of the cell, at a testosterone concentration of 10^{-9}M, after which there is at the plateau an inhibitory effect at 10^{-5}M. Do you have some data on the transformation of testosterone and dihydrotestosterone in these 2 situations?

King. Not at the two concentrations. At 0.5 nM, testosterone can be converted to DHT but I think the data favour the view that testosterone can be active in its own right (Gordon, Smith and King: *Molec. cell. Endocr.* **1** (1974) 259–270). This conclusion is based on the observation that after addition of [^{3}H]-testosterone to the culture

medium the major steroid in the nucleus is [^{3}H]-testosterone with much lower levels of [^{3}H]-DHT.

Lippman. Roger, I agree with the points that you made. With cell cultures one can separate a variety of serum regulating co-factors, as you said, but I think that one must always reserve the idea that cells that are in culture may be very highly selected and their regulation may, in fact, be different from the *in vivo* situation. By that I mean specifically, if you take prostate *in vivo* and castrate the animal obviously there is a massive loss of cell mass and a lot of cell death and killing. But the cell simply stops growing or stops growing very slowly which obviously can be resuscitated by re-administration of androgen. That's a very different picture from what you observe in a sort of homogenous lone cell population where you withdraw androgen and re-administer it. You don't see this sort of 2 phase kind of response and I think you have to wonder whether or not there are 2 different kinds of populations of cells *in vivo* or what the explanations for that kind of difference are and whether or not they spring from selection of the given cell line that one is using in culture.

King. You are correct; the point I was trying to make is that in the simplified conditions pertaining in culture one may be observing effects of steroids that are not complicated by the many other factors influencing organ/tumour size in an intact animal.