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Steroids, Growth Factors and The Prostate

STEROIDS AND THE PROSTATE

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Summary—Interelationships between steroid and growth factor regulation of cell proliferation has been examined in two androgen sensitive prostatic cell lines, grown in defined medium. The cell lines used were derived from normal (CAPE) and neoplastic (LNCaP) tissues. The growth of both cell lines was elevated by challenge with serum, androgens and epidermal growth factor (EGF) used as single agents. The effects of androgen in CAPE were small, but significant while the profound effects of these agents on the growth of LNCaP were confirmatory of other studies. Androgens upregulated EGF receptor expression in LNCaP measured by both ligand binding capacity and mRNA analysis. This was not observed in the CAPE cells. Addition of serum (whole or charcoal stripped) suppressed the observed androgenic stimulation of EGF receptor expression in LNCaP. This apparent anomaly is discussed in relation to the growth enhancing properties of serum in these cell lines and in the wider context of normal and neoplastic growth control in the prostate.

INTRODUCTION

Growth and the development of adult differentiated function in the prostate gland involves processes critically dependent on the presence of circulating androgens. The major circulating androgen in the male is testosterone of which 90–95% is testicular in origin [1]. This steroid is metabolised to 5α-dihydrotestosterone (5α-DHT) in the prostatic tissues of most species and it is predominantly this androgen which interacts with prostatic androgen receptors, cellular proteins which mediate the specific activities of this class of steroid in the prostate [2]. Deficiencies in either the enzyme activity (5α-reductase) required to produce 5α-DHT or in androgen receptor expression in prostatic cells results in only rudimentary prostatic growth during development [3, 4]. Similarly depletion of androgens from the circulation of experimental animals by orchidectomy results in a severe reduction in prostatic size and cellular content [5]. Normal prostatic size and function can be restored by administration of exogenous androgen to these animals.

Prostatic cancers in both cancer patients and in experimental systems appear to retain similar dependencies on androgenic steroids. In patients, 70–80% of prostatic cancers are, at least initially, profoundly responsive to androgen ablation therapy, achieved either by bilateral orchidectomy or chemical castration [6]. In either case, median duration of response is approx. 2 years, with average survival following relapse limited to only 6 months. Progressive disease is characterized by an increasing predominance of tumour cell populations for which the requirement for androgens to sustain growth is either reduced or absent. The transition from androgen dependent to independent growth in prostatic tumours is attributed to intrinsic heterogeneity in cellular responsiveness to androgen ablation at the outset of therapy, insensitive cells being able to continue growing and perhaps favoured by the selection pressure of androgen withdrawal. More generally these observations suggest that while the effects of androgens are clearly significant in normal prostatic physiology, other influences, potentially reflective of those which sustain the growth of hormone independent tumours, may play a more fundamental role in prostatic growth control.

Of the other known circulating agents, oestrogens have been most convincingly linked with prostatic growth abnormalities [7, 8]. The change in plasma oestradiol/testosterone, ratios caused by the slow decline in free testosterone levels that occurs with age in the human male

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have been correlated with the incidence of onset of benign prostatic hyperplasia (BPH). BPH has been induced in the dog by co-administration of androgens and oestrogens [7]. Some of the effects of estrogens on the prostate are indirectly mediated through actions at the hypothalamic and pituitary level, but others are clearly intraprostatic in particular on the proliferation and synthetic activities of fibromuscular stroma [8]: cellular receptors for oestrogens and glucocorticoids have been demonstrated in both prostatic tissue and in prostatic cell lines ([9, 10] and Dr P. Davies, personal communication) and the latter steroids have been shown to retard some of the effects of castration in experimental animals [11]. This phenomenon and recent analysis of steroid hormone receptor–DNA interactions have provided some evidence for potential interregulation of steroid responsive genes by the various classes of receptors under certain circumstances [12].

Studies of the developmental biology of the urogenital-sinus and its associated structures, including the prostate, have provided evidence for intraglandular growth control processes involving induction signalling between stroma and epithelium [13]. While the mediating factors in these interactions have yet to be definitively identified they potentially belong to established classes of signalling systems. These include polypeptide growth factors and several classes of these agents have been associated with prostatic tissues [14–18]. In particular members of the fibroblast growth factor (FGF) family have been identified as present in prostatic extracts [14] or shown to stimulate the proliferation of prostatic cells [15]. Similarly, factors whose activities are mediated through the epidermal growth factor (EGF) receptor have been implicated in prostatic growth processes [16]. In addition the epithelial growth inhibitor, transforming growth factor β(TGFβ) has been identified in prostatic extracts [17]. Receptors for all these agents have been identified in prostatic cells [18–20]. It would seem therefore that there are a number of known growth modulators present within the prostate gland, available for intercellular signalling. It is likely that the activities of these agents are modulated by systemic factors such as steroids and that a complex network of interactions between the effects of various agents is required to provide normal glandular homeostasis. Conversely this implies that there is a wide potential for variations leading to aberrant growth patterns.

In the present study we have examined the activities of EGF in two androgen sensitive cell lines, one of normal canine origin (CAPE) and the other derived from a human carcinoma (LNCaP). We have examined the effects of androgens and EGF on cell proliferation in serum free medium and made a preliminary evaluation of the effects of androgens on EGF receptor content and capacity for response to EGF in these cell lines.

EXPERIMENTAL

Cell lines

Epithelial cell lines CAPE 1 and LNCaP were used in this study. Both cell lines have been shown to contain receptors for androgens [21, 22] and their growth modulated by these steroids [23, 24].

Cells were maintained in monolayer cell culture in serum supplemented medium for routine passaging, Dulbecco's Modified Eagle's medium (DME) for CAPE and RPMI 1640 for LNCaP (Gibco Ltd., Paisley, Scotland). Where indicated cells were grown in a serum free formulation, DCCM-1 (Biological Industries, Cumbernauld, Scotland) modified by the exclusion of insulin.

Cells were passaged by disruption of monolayers by exposure to trypsin/EDTA (0.05/0.02% w/v) in PBS (Dulbecco A) (Flow Labs, Rickmansworth, Herts.), resuspension of cells in medium and inoculation of culture flasks and plates at various cell densities.

Growth analysis

Proliferation rates of cell lines challenged with various agents were evaluated in large populations during exponential growth and through growth curves, and by clonal growth analysis in monolayers. In all experiments cells were inoculated into dishes in serum supplemented (5%, v/v) medium. On the first day after inoculation cells were washed 3× with PBS and treatments applied. In experiments using large populations 24 well plates were seeded at a density of 5000 cells/well. In clonal growth analyses 60 mm dia dishes were seeded with 1000 cells/dish. Growth rates were evaluated by Coulter Counter in suspension or image analysis (clonal growth experiments).

Receptor analysis

Receptor determinations were performed using whole cells grown in 96 well culture plates (Falcon Plastics, Becton & Dickinson U.K.

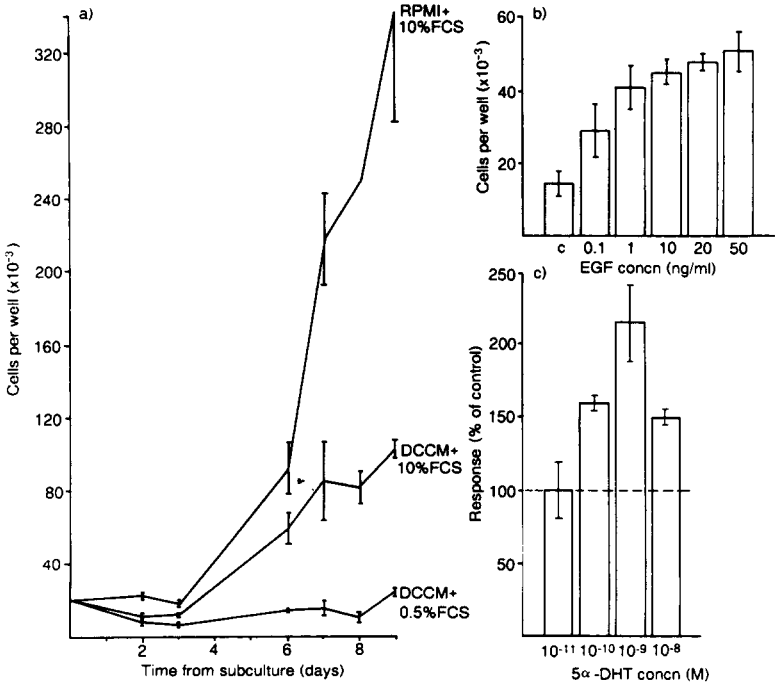

Fig. 1. Summary of growth characteristics of LNCaP: (a) comparative growth curves of cells grown in RPMI 1640 or DCCM1 medium supplemented with whole serum; (b) titration of the effects of EGF during rapid growth, (DCCM1, serum free) and (c) titration of the effects of 5α-DHT (DCCM1, serum free). Data represent means ($n = 3$–6) ±SD. Significant elevation of growth was observed with EGF (1–50 ng/ml) and 5α-DHT (10^{-10}–10^{-9} M) ($P = <0.005$).

Ltd., Oxford, England). In binding assays cells were incubated with [^{125}I]EGF in the presence and absence of 100-fold molar excess of unlabelled competitor in assay buffer [RPMI 1640 medium + 0.1% bovine serum albumin (BSA)] [Boehringer Corporation (London) Ltd., Lewes, Sussex, England] for 2 h at 4°C. Total incubation volume was 20 μl. After incubation, cells were either washed directly with assay buffer (5 × 200 μl) (CAPE) or released from culture wells by vigorous pipetting and transferred to glass fibre filters prior to washing to remove unbound ligand (LNCaP). Bound radioactivity in cell preparations (solubilized with 0.1 M NaOH) or on filters was evaluated by γ counting. Results were analysed by Scatchard plot [25] and curved data resolved into linear components by the methods of Rosenthal [26] and Chamness and McGuire [27].

RNA isolation and analysis

Total RNA preparations were made by a method similar to Davis *et al.* [28]. Briefly, cultures of LNCaP challenged with various treatments during rapid growth were lysed directly in culture dishes by exposure to 4 M guanidinium isothiocyanate in 0.3 M sodium acetate buffer containing β-mercaptoethanol (8.35 ml/l). The resultant lysate was resolved by centrifugation through caesium chloride and RNA and DNA separately isolated. RNA was further purified by phenol extraction and ethanol precipitation and stored at −70°C. RNA preparations were resolved in 1% agarose gels (20 μg/lane) and capillary blotted onto nitrocellulose filters. Filters were dried at room temperature, baked for 2 h at 80°C, and prehybridized overnight at 42°C in prehybridization buffer [20 mM Tris–HCl pH 7.4, 4 × SSC, 40% formamide (v/v) 20 μg/ml denatured salmon sperm DNA, 1 × Denhardt's (Denhardt's 100× : 2% polyvinylpyrolidone, 2% BSA, 2% Ficol 400 w/v)]. Filters were hybridized in the same buffer as used for prehybridization with the addition of 1–10 × 10^6 cpm/ml [^{32}P]labelled cDNA probe overnight at 42°C. Filters were then washed at high stringency (final wash: 0.25 × SSC + 0.1% SDS at 55°C, 30 min) air dried and autoradiographed.

RESULTS

Growth responses; effects of serum, androgen and EGF

Both cell lines were maintained in DCCM1 in the absence of serum [Figs 1(a) and 2(c)], but

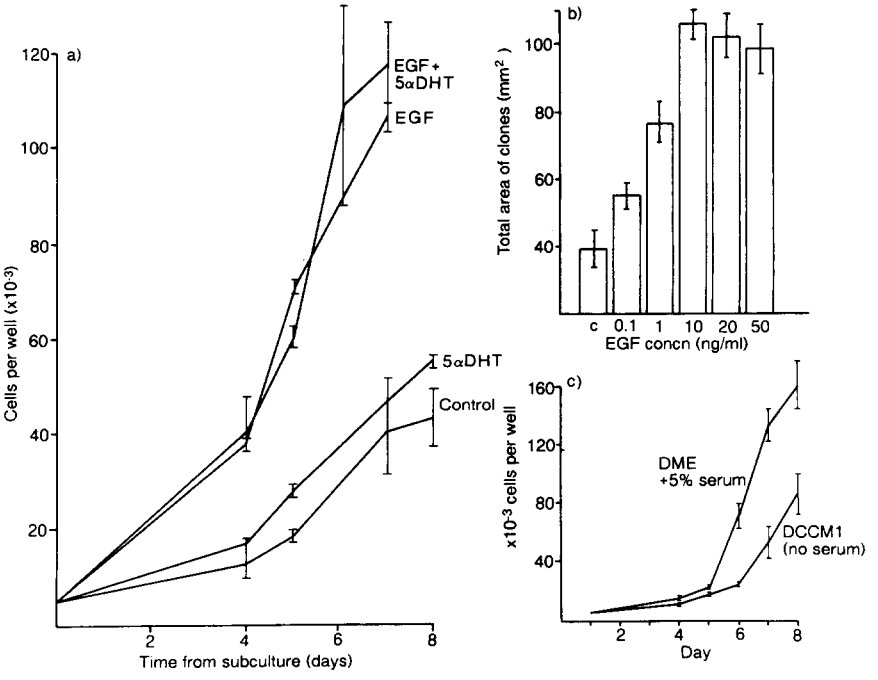

Fig. 2. Summary of the growth characteristics of CAPE: (a) comparative growth curves of cells grown in DCCM1 medium (serum free) supplemented with 5α-DHT or EGF alone or in combination; (b) titration of the effects of EGF in clonal growth assays, (DCCM1, serum free) and (c) comparative growth curves of cells grown in either DME supplemented with whole serum or in DCCM1 (serum free). Data represent means ($n = 6$) $\pm$SD. Significant elevation of growth was observed with EGF (1–50 ng/ml) ($P = <0.005$) and with 5α-DHT (10^{-9} M) during exponential growth.

neither grew as well as in conventional serum supplemented formulations. The CAPE cell line actively proliferated in DCCM1 and performed sigmoidal growth curves in this medium although log phase doubling times were rather longer (>50 h) and maximum cell densities reached were lower than in serum or growth factor supplemented cultures. The LNCaP cells were maintained in DCCM1 with little or no growth over a 10-day period.

Proliferation in both cell lines was significantly increased by the presence of serum when added as a medium supplement [Figs1(a) and 2(c)]. The effect of serum was dose dependent, optimum concentrations in CAPE and LNCaP being 5 + 10%, respectively in standard medium formulations (titrations not shown). In DCCM1 the effects of serum were less pronounced with concentrations <1% showing no significant growth enhancing activity over controls (DCCM1 alone) in either cell line. The proliferations of both cell lines was significantly enhanced by the presence of androgens during log phase growth in the absence of serum [Figs 1(c) and 2(a)] although the effects in LNCaP were greater than that observed in CAPE at all concentrations. A concentration of 10^{-9} M was optimally effective in each cell line.

EGF was a potent mitogen in both cell lines measured by both growth curve analyses and clonal growth assay [Figs 1(b) and 2] and maximally effective at a concentration of 10 ng/ml. In a series of combination experiments using the CAPE cell line the effects of androgen were not enhanced by the presence of EGF [Fig. 2(a)] or charcoal stripped serum (data not shown).

EGF receptor analyses

Saturation analyses using radioiodinated EGF in the presence and absence of competitor produced curved Scatchard plots in experiments with both cell lines [29]. These were resolved into low and high affinity components using a combination of mathematical and graphical methods. Low affinity EGF binding was elevated in rapidly growing LNCaP cultures maintained in the presence of 5α-DHT [Fig. 3(b)]. The effects of this androgen on high affinity binding were less marked [Fig. 3(a)]. No differences were observed in either high or low affinity binding between androgen supplemented and control cultures during rapid growth in CAPE cells, however significant depression of high affinity EGF receptor content occurred with the transition between logarithmic and confluent growth phases. This effect was enhanced by the

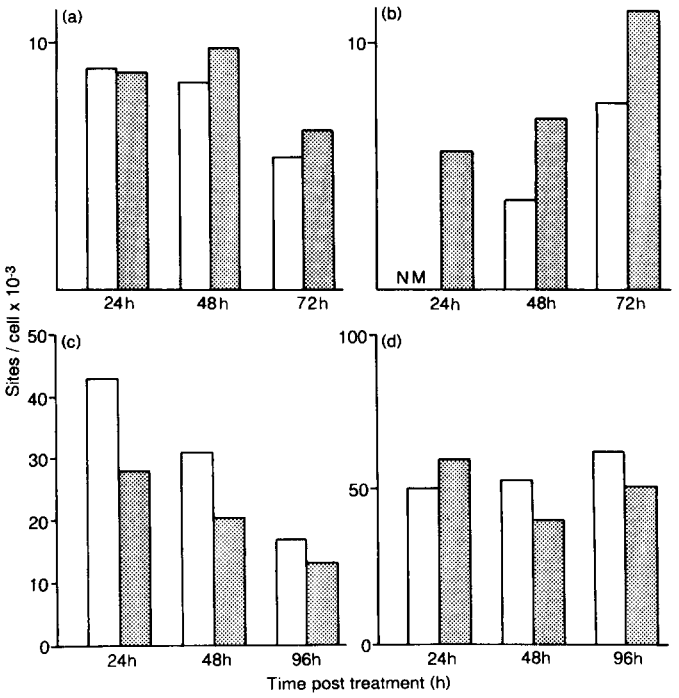

Fig. 3. Comparison of EGF receptor content in LNCaP [(a) and (b)] and CAPE cells [(c) and (d)] in the presence (▦) and absence (□) of 5α-DHT. High affinity [(a) and (c)] ($K_d = 2–5 \times 10^{-10}$ M) and low affinity [(b) and (d)] ($K_d = 2–6 \times 10^{-9}$ M) receptor content is presented.

presence of 5α-DHT [Fig. 3(c)]. Effects of androgen on low affinity binding were less marked.

Northern blot analyses

Evaluation of LNCaP RNA by Northern blot hybridization using [^{32}P]labelled EGF receptor cDNA identified a prominent transcript of 8.5–10 kb consistent with that observed in other cell types [30]. This transcript appeared to be upregulated by androgen and downregulated by serum, relative to controls, at various time points post-treatment (Fig. 4). The effects of 5α-DHT were maximal at the 6 and 24 h time points while stripped serum suppressed transcript levels at all time points and abrogated the effects of DHT at the 6 and 24 h time points (Fig. 4).

DISCUSSION

In the present study, general growth characteristics and responses to EGF, androgen and serum have been compared in two prostatic, androgen sensitive cell lines of normal and neoplastic origins. The CAPE cells performed sigmoidal growth curves and cell densities did not exceed 70×10^3 cells/cm^2, even under the most favourable conditions, and were always monolayered. In contrast, the LNCaP cells could be followed up to densities of at least 200×10^3 cells/cm^2 in the presence of serum and there was no discernable slowing of growth rates even at these densities. Supplementation of CAPE cultures with EGF alone produced growth rates that were not significantly different from serum supplemented standard medium formulations. This was not the case with LNCaP cells in which serum supplementation was significantly more effective than other treatments either used as single agents or in combinations.

The effects of 5α-DHT on the growth of LNCaP cells were highly significant at concentrations between 10^{-10} and 10^{-8} M. The effects on CAPE cells were small but significant at nanomolar concentrations during logarithmic growth, and were not enhanced by EGF. EGF was a potent mitogen in both cell types and was maximally effective at similar concentrations (10 ng/ml). Elevated growth rates in cultures of LNCaP cells supplemented with androgens have been reported previously [24]. In these studies cells were challenged in charcoal stripped serum in standard (RPMI 1640) medium formulations. Similar biphasic dose responses were observed with androgens in these studies, however the concentration with maximal growth enhancing effects was an order of magnitude

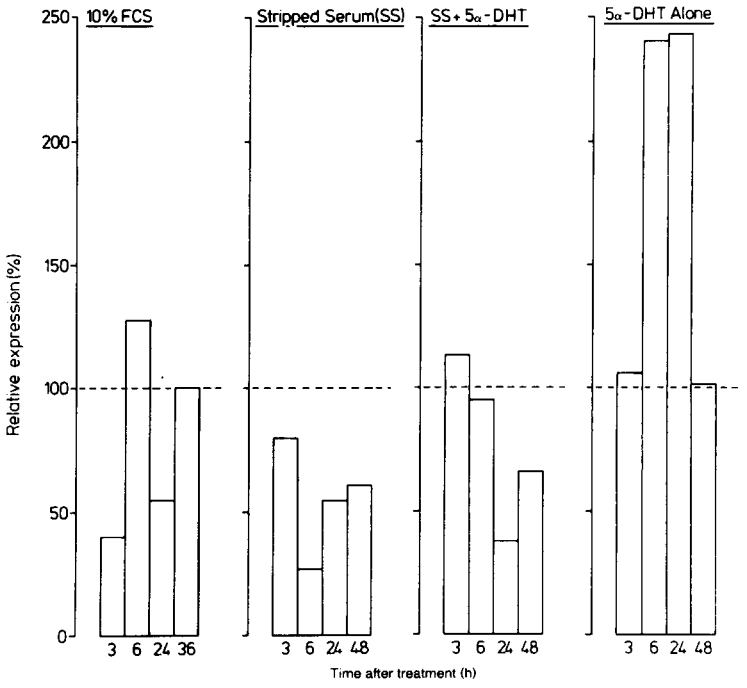

Fig. 4. Comparison of EGF receptor mRNA expression in LNCaP cells challenged with 5α-DHT and whole or charcoal stripped (SS) foetal calf serum (FCS). The effects of treatments are presented relative to control and are based on optical density measurements of Northern blots of an EGF receptor mRNA of 8.5–10 kb. Equal loading of lanes (20 μg total RNA) was confirmed by reprobing of blots with an actin cDNA.

lower than that observed in the present study. This discrepancy may be due to complex interactions between androgens and the activities of serum factors. The present data would tend to favour some form of synergy with serum derived mitogens rather than effects on suppression of growth inhibitory serum factors suggested as a mode of action for androgens in LNCaP cells elsewhere [31].

The relatively low levels of growth enhancement observed in the CAPE cells in response to androgens was a finding similar to that reported previously with other epithelial cell lines derived from normal prostatic tissue [32]. These studies suggested that the critical dependence of adult prostatic epithelial growth and function on circulating androgens apparent in *in vivo* studies may be mediated by androgenic regulation of inductive signalling between prostatic cell types, possibly similar to that demonstrated during foetal development [13]. EGF may be involved in this kind of intraglandular signalling system either as a constantly present agent or as a temporal factor induced under conditions of glandular growth during development and regrowth following castration/readministration of androgens. Peptides in the EGF family could be produced by epithelial or stromal components and an interactive framework for growth factor synthesis in the developing prostate has been recently postulated [33].

Ligand binding analyses using whole cells of both cell types produced curved Scatchard plots indicative of two classes of binding site of high ($K_d = 2\text{–}5 \times 10^{-10}$ M) and lower ($K_d = 2\text{–}6 \times 10^{-9}$ M). This was essentially similar to data derived from saturation analyses using membrane preparations from these cells. This finding in respect of LNCaP is in contrast to other studies [24] in which only one site was described. The discrepancy between the present data and that of others would appear to be a function of variation in methodologies and analysis procedures in what is a complex assay system. Our data provides some additional information concerning the EGF receptors and their interaction with other growth modulators. A number of studies have suggested that the transition between low and high affinity states in the EGF receptor system has functional significance, with low affinity being associated with monomeric inactive forms and high affinity being indicative of oligomerized, functionally activated receptors [34–36]. The upregulating activity of androgen on EGF receptors in LNCaP was largely on low affinity binding

which suggests effects on monomeric, potentially newly synthesized receptor. High affinity binding was not significantly affected indicating that the effects of androgen were probably not on receptor oligomerization and activation. This observation is in part confirmed by the Northern blot analyses which demonstrate increased levels of EGF receptor mRNA in LNCaP cells treated with androgens (Fig. 4). The activities of androgens on EGF receptor in CAPE cells were confined to confluent growth phase, when 5α-DHT appeared to suppress high affinity receptor content during a time when levels of this protein were declining in these cells. These results suggest that in these cells, derived from normal tissue, androgens may be more generally involved in receptor activation and may mediate some aspects of response to EGF during growth phases where proliferative activity was reduced, possibly in favour of more differentiated functions [21]. How this may occur is not clear, but the absence of effects on low affinity binding may indicate that the EGF receptor gene induction is probably not involved. Generally, the results suggest that androgens and EGF receptors are less functionally linked to the proliferative process in CAPE than in LNCaP.

The effects of whole and charcoal stripped serum on the growth and EGF receptor expression in LNCaP were anomalous when viewed in relation to the overall potent growth enhancing effect of this supplement and in the context of the activities of androgens on EGF receptor content. It might have been considered that at least some of the growth enhancing properties of whole serum were due to endogenous presence of androgens yet serum supplementation did not increase EGF receptor expression in LNCaP. Charcoal stripped serum appeared to suppress EGF receptor expression and abrogate the upregulatory effects of androgen on this parameter, both in this study and in previous ligand binding based analyses [29]. These observations may be a secondary phenomenon related to autocrine ligand regulation since EGF has been shown to upregulate its receptor [37] and may potentially compete for radioiodinated ligand in assay systems. However, studies of EGF and related peptide expression in LNCaP cells suggest that the production of these proteins is unaffected by challenge with androgens [38]. Taken together these studies indicate that high EGF receptor content *per se* is probably not the major controlling influence in the proliferation of this cell line although subtle modulation of this protein may be important.

The apparent absence of androgen response elements associated with the EGF receptor gene suggest that the effects of androgens on receptor regulation are probably secondary to activities on other genes most notably those of transcription factors which are modulated by steroid and serum sensitive regulatory elements [39, 40]. Analyses of EGF receptor gene transcription control in *in vivo* and *in vitro* assay systems have suggested the involvement of proteins similar to the SV40 transcription factor Spl [41] which appear to act co-ordinately with other transcription factors as yet only partially identified [42]. These may include members of the Apl/jun family and the EGF receptor gene promoter region does contain a sequence with high homology to that described for recognition and binding by these transcription factors [29, 43]. The latter act in so called "leucine zipper" associations with the cfos gene product in the process of transcription control [43] and the upregulation of this protein appears to be an important early event in cellular responses to androgens [44].

It is clear that the involvement of androgens in growth factor pathways and cell proliferation is far from simple. The divergence observed between cell lines in the present study between the effects of androgens on EGF receptor expression probably reflects complex interactions between these steroids and their receptors and a number of interdependent genes. The recent anomalous growth enhancement observed in LNCaP when challenged with antiandrogen [45] and the reported mutation in the LNCaP androgen receptor [46] suggest additional variability in the genes potentially regulated by distinct forms of androgen receptors present in different cell types. While it is tempting to equate androgen induced elevation of EGF receptor levels with increased proliferation rates and by inference identify this as a basic growth modulating phenomenon, a more detailed evaluation of androgenic regulation of genes more centrally involved in transcription control would seem to be essential. Studies of this type may well identify processes more fundamental to proliferation and assign aspects such as relative levels of growth factor receptor expression as of at least secondary or even incidental importance in growth processes.

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