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Progression to Steroid Autonomy is Accompanied by Altered Sensitivity to Growth Factors in S115 Mouse Mammary Tumour Cells

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Progression to steroid autonomy is a major clinical problem in the treatment of steroid-sensitive tumours. Molecular mechanisms remain unknown but recent hypotheses imply a role for growth factors in this progression. Since S115 + A androgen-responsive mouse mammary tumour cells provide a model system to study this phenomenon *in vitro*, we have used this model to investigate growth factor gene expression and sensitivity during progression from a steroid sensitive to insensitive state. S115 + A androgen-responsive cells showed a positive proliferative response, morphological response and increased saturation density to various forms of fibroblast growth factor (FGF) and transforming growth factor beta (TGFβ) in both monolayer and suspension culture. A marked synergy was noted, however, between FGF and TGFβ in promoting growth in suspension culture. S115 + A cells possessed mRNA for both acidic FGF (aFGF) and TGFβ$_1$, both of which were increased by testosterone. Progression to androgen insensitivity was associated with a reversal of growth factor response such that all growth factor responses became generally inhibitory on growth of the unresponsive cells but with a particularly striking synergistic action between FGF and TGFβ$_1$ on inhibition of both monolayer and suspension growth. Levels of aFGF and TGFβ$_1$ mRNAs remained low in steroid-insensitive S115 − A cells, indicating that loss of response was not associated with any constitutive upregulation of endogenous production of one of these growth factors. The scientific and clinical implications are discussed.

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INTRODUCTION

Steroid hormones regulate the growth of many normal and tumour cells including cells of breast and prostate. The molecular mechanism is known to involve interaction of the steroid hormone with an intracellular receptor which then acts as a ligand-activated transcription factor [1]. However, much less is known about the nature of the target genes in the cell growth response. In recent years, it has been proposed that steroid regulation of cell growth could be mediated by altered production of or sensitivity to growth factors. There is considerable evidence demonstrating that specific growth factors are mitogens for breast [2] and prostate [3, 4] cells but the extent and nature of the interaction between steroid hormone and growth factor regulatory pathways remains to be clarified.

Growth factor studies in several cell lines have suggested that androgen sensitivity may be associated with a positive response to and/or production of fibroblast growth factors (FGF) [5–9]. Clonal cell lines derived from the Shionogi 115 spontaneous mouse mammary carcinoma [10] including S115 + A cells [11] and SC3 cells [9] have provided model systems for studying mechanisms involved in androgen regulation of cell growth. Morphology and proliferation of these cells are regulated in anchorage-dependent and anchorage-independent culture by androgen and glucocorticoid [11, 12]. More recently, androgen has been shown to induce in SC3 cells secretion of a heparin-binding growth factor of the FGF family [13] which can

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bind to an FGF receptor present on the cells [9, 14] and which is a variant form of the FGF receptor type I (FGFR-I) [15]. SC3 cells have been shown to be sensitive to exogenous basic FGF (bFGF) [16], and furthermore, cell regulation by androgen, glucocorticoid and bFGF are all inhibited by a bFGF neutralizing antibody [17]. This would suggest that steroid regulation is mediated, at least in part, in these cells through induction of an FGF-like peptide acting in an autocrine mode via an FGF receptor. This FGF-like peptide has now been characterized as a novel member of the FGF family named androgen-induced growth factor (AIGF) (int 8) [18] and transfection experiments confirm that it can act on cells in an autocrine loop via the variant FGFR-I [9]. In early stages of androgen stimulation, AIGF is associated with glycoaminoglycan in the extracellular matrix [9, 19] and androgen action can be blocked by heparin [20]. In this respect, it is interesting that the steroid induced alteration in cell morphology in S115 + A cells is also related to suppression of syndecan expression [21, 22], since syndecan is a cell surface proteoglycan which is known to interact with FGF [23] and can modulate its response [24].

A major problem in the clinical treatment of steroid-sensitive tumours is the inevitable progression of the tumour cells to a state of steroid insensitivity resulting in failure of endocrine therapy. The S115 + A cell line provides an *in vitro* model system to study mechanisms involved in this progression since these cells were cloned from a tumour which progresses *in vivo* from a steroid sensitive to insensitive state [25] and this progression can be mimicked *in vitro* [11, 26, 27]. Growth of the steroid responsive S115 + A cells in the long-term absence of steroid results in the cells becoming unresponsive (S115 − A cells) to either androgen or glucocorticoid in monolayer [11, 26] or suspension [11, 27] culture. The mechanism involves an increased growth rate in the absence of steroid with no alteration to the steroid-stimulated growth rate, and occurs in an ordered, reproducible series of phenotypic changes [11, 26, 27] but with no loss of steroid receptor number or function [28]. Recently, it has been generally postulated that uncoupled growth factor production could provide a mechanism for such non-receptor-mediated events. Steroid independence is suggested to result from an alteration to either production of growth factor or sensitivity to growth factor. Such alterations may or may not be related to those pathways normally involved in steroid regulation of growth of the steroid-sensitive cells. This manuscript describes growth factor gene expression and sensitivity as S115 + A cells progress from a state of steroid sensitivity to one of steroid insensitivity.

EXPERIMENTAL

Nomenclature of S115 cells

Stock S115 + A cells are a clonal cell line maintained in androgen that exhibit a positive proliferative response to androgen. Long-term maintenance of such cells in the absence of androgen results in the cells becoming unresponsive to androgens, and such cells are then called S115 − A cells. Experimental growth of S115 cells in the presence or absence of testosterone is indicated as + T or − T, respectively.

Culture of stock S115 cells

Stock S115 + A cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% foetal calf serum (FCS) (Gibco, Glasgow, Scotland), 40 mM HEPES buffer (Sigma, Poole, England) and 3.5×10^{-8} M testosterone (Steraloids, Croydon, England). Testosterone was dissolved in ethanol and added such that the final concentration was 0.001% ethanol in culture medium. Cells were seeded at 0.1×10^5 cells/ml in 16 ml aliquots in 9 cm plastic tissue culture dishes (Nunc, Denmark) and placed in a humidified atmosphere of 10% carbon dioxide in air at 37°C.

Cells were subcultured at weekly intervals. Cells were suspended by treatment in 5 ml of 0.06% trypsin/0.02% EDTA (pH 7.3) and added to 5 ml of culture medium. The cells were pelleted by centrifugation, resuspended in culture medium alone, counted on a haemocytometer and replated as above.

Stock S115 − A cells were grown as for + A cells except that testosterone was omitted from the culture medium and serum was stripped with dextran-charcoal (DC-FCS) [29].

Cell growth experiments

For monolayer culture, cells were suspended from stock plates by treatment with 5 ml 0.06% trypsin/0.02% EDTA (pH 7.3), added to 5 ml DMEM/2%DC-FCS/40 mM HEPES buffer and counted on a haemocytometer. Cells were added to the overall required volume of medium DMEM/2%DC-FCS/40 mM HEPES buffer at a density of 0.1×10^5 cells/ml and plated in monolayer in 2.45 ml aliquots into 35 mm plastic tissue culture dishes for 24 h. The medium was then changed to contain the required concentration of serum, steroid or growth factor. Stock solutions of growth factors were made as follows: bovine pituitary fibroblast growth factor (bFGF) (Flow, Irvine, Scotland) at 10 μg/ml in water; bovine recombinant basic fibroblast growth factor (rbFGF) (Boehringer Mannheim, Germany) at 10 μg/ml in water; bovine brain endothelial cell growth factor (ECGF) (Boehringer Mannheim, Germany) at 1 mg/ml in water; human recombinant acidic fibroblast growth factor (raFGF) (Bachem, CA, U.S.A.) at 10 μg/ml in water; bovine pancreatic insulin (ins) (Sigma, Poole, England) at 10 mg/ml in 6 mM HCl; epidermal growth factor (EGF) (Flow, Irvine, Scotland) at 10 μg/ml in water; porcine transforming growth factor beta 1 (TGFβ1) (R&D systems, Minneapolis, U.S.A.) at

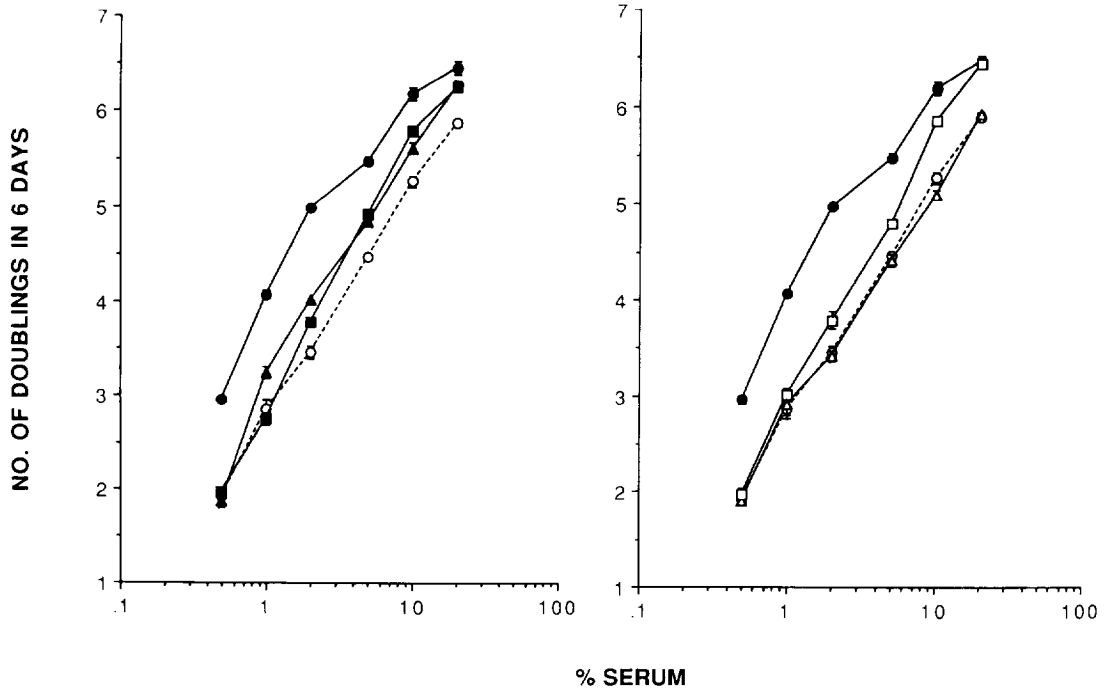

Fig. 1. Effects of growth factors on monolayer growth of androgen responsive S115+A mouse mammary tumour cells at different serum concentrations. Cells were grown without steroid or growth factor addition (○) or with 3.5×10^{-8} M testosterone (●), 10 ng/ml rbFGF (▲), 10^{-10} M TGFβ_1 (■), 100 ng/ml EGF (□), 1 μg/ml insulin (△). Cell growth was expressed as the mean number of cell doublings in 6 days and error bars show the standard error from triplicate estimates (where no bars are shown, variation was too low for visual display). Significance of effects of each growth factor at each serum level are indicated by *P* values obtained by comparison of triplicate values for each growth factor as compared to triplicate values in the absence of growth factor at the same serum level as follows: 0.5% serum + Ins 0.82, +TGFβ 0.59, +EGF 0.60, +rbFGF 0.12; 1% serum + Ins 0.58, +TGFβ 0.14, +EGF 0.08, +rbFGF 0.004; 2% serum + Ins 0.56, +TGFβ 0.01, +EGF 0.005, +rbFGF 0.004; 5% serum + Ins 0.54, +TGFβ 0.006, +EGF 0.004, +rbFGF 0.002; 10% serum + Ins 0.10, +TGFβ 0.01, +EGF 0.001, +rbFGF 0.015; 20% serum + Ins 0.41, +TGFβ 0.008, +EGF 0.002, +rbFGF 0.007.

12.5 μg/ml in 4 mM HCl/1% bovine serum albumin. Culture medium was changed routinely every 3–4 days.

For suspension culture, cells were grown in the same medium as for monolayers but in 35 mm plastic bacteriological dishes (Sterilin, Teddington, England) to which the cells did not attach.

Cell counting

Cells in monolayer were washed with isotonic saline *in situ*. Cells in suspension were harvested in saline and pelleted by centrifugation. Cells were then lysed in 2 ml 0.01 M HEPES buffer/1.5 mM magnesium chloride plus 4 drops of zaponin (Coulter Electronics, Harpenden, England) for 5 min (monolayers) or 1 h (suspensions). The nuclei released were counted in Isoton (Coulter Electronics) in triplicate on a model ZB1 Coulter counter. All cell counts were done on triplicate dishes and results were calculated as the mean number of doublings in a specified number of days ± standard error [26]. *P* values (two-tail) were calculated using the Student *t*-test two sample assuming unequal variances (using Microsoft Excel for IBM PC).

Preparation and analysis of RNA

A minimum of 3 × 15 cm dishes of stock monolayer cultures were used for each RNA preparation. Cells were washed *in situ* with phosphate-buffered saline (PBS), harvested with a rubber policeman into ice-cold PBS and pelleted by centrifugation. Whole cell RNA was prepared by the guanidinium-caesium chloride method [30].

For Northern blotting, RNA was subjected to electrophoresis in 1.5% agarose-formaldehyde gels, transferred to Hybond-N (Amersham International, England) by blotting in 20 × standard saline-citrate buffer (SSC) and hydridized to 10^6 cpm of ^{32}P-labelled DNA probe per ml. Hybridization was in 5 × SSPE, 5 × Denhardt's 50% formamide, 0.5% SDS, 20 μg/ml salmon sperm DNA at 42°C for 18 h. DNA was labelled with ^{32}P by random priming kit (Amersham International, England) and blots were washed at a stringency of 0.1 × SSPE, 0.1% SDS for 30 min at 65°C. DNA probes used were a 280 bp fragment of human TGFβ1 in pSP72 [31] and a 2.3 kb PstI fragment of mouse β-actin (kindly provided by Dr K. Willison). All cloned sequences were cut out and isolated from plasmid before use.

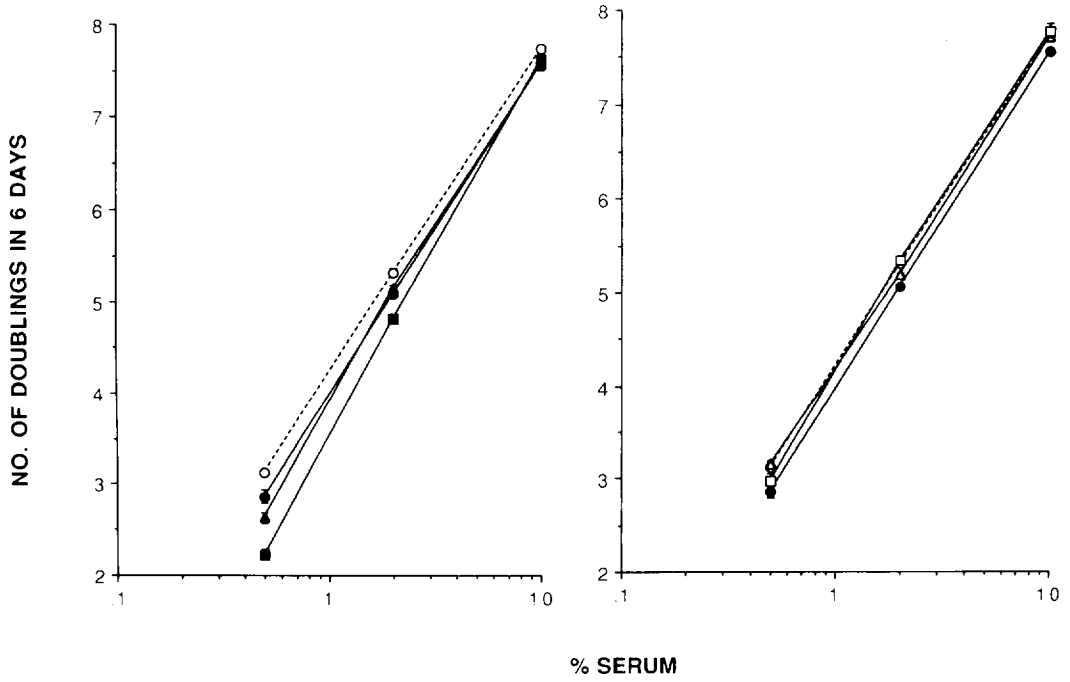

Fig. 2. Effects of growth factors on monolayer growth of androgen insensitive S115-A mouse mammary tumour cells at different serum concentrations. Cells were grown without steroid or growth factor addition (○) or with 3.5×10^{-8} M testosterone (●), 10 ng/ml rbFGF (▲), 10^{-10} M TGFβ_1 (■), 100 ng/ml EGF (□), 1 μg/ml insulin (△). Cell growth was expressed as the mean number of cell doublings in 6 days and error bars show the standard error from triplicate estimates (where no bars are shown, variation was too low for visual display). Significance of effects of each growth factor at each serum level are indicated by *P* values obtained by comparison of triplicate values for each growth factor as compared to triplicate values in the absence of growth factor at the same serum level as follows: 0.5% serum + Ins 0.41, +TGFβ 0.004, +EGF 0.22, +rbFGF 0.015; 2% serum + Ins 0.10, +TGFβ 0.001, +EGF 0.63, +rbFGF 0.03; 10% serum + Ins 0.72, +TGFβ 0.20, +EGF 0.63, +rbFGF 0.11.

Ribonuclease protection assays were performed exactly as described previously [31]. The aFGF riboprobe was synthesized from a 480 bp fragment of mouse aFGF which contained exclusively sequences for the coding region of the gene with 9 nucleotides of coding at both 5′ and 3′ ends lacking and cloned into pGEM4 at SacI-XbaI sites of the polylinker (kindly provided by Dr C. Dickson). The plasmid was cleaved with EcoR1 and transcribed with T7 polymerase. Mouse aFGF mRNA protects 480 bp of the 516 bp probe (personal communication, Dr C. Dickson). The γ-actin riboprobe was transcribed from a HinfI-digested actin cDNA clone in pSP64 using SP6 polymerase. Mouse γ-actin mRNA protects a 75 bp probe fragment [31].

RESULTS

Growth factor regulation of anchorage-dependent growth

Growth rate of androgen-responsive S115 + A cells increased with increasing serum concentration both in the presence and in the absence of testosterone (Fig. 1). However, the growth rate remained greater in the presence of testosterone at all concentrations of serum. The growth response of the cells to various exogenous growth factors was then investigated at different serum levels (Fig. 1). S115 + A cells responded positively with increased growth rates to bFGF, EGF and TGFβ_1 but these responses were markedly affected by serum concentration. Greater effects were found for all three growth factors at higher serum levels with no effects visible at all at 0.5% serum. No response to insulin (up to 1 μg/ml) was found in these cells at any serum level from 0.5 to 20%. Experiments in 1 and 2% serum have been carried out three times to check for reproducibility.

S115 + A cells showed a positive proliferative response to various forms of bFGF and aFGF in a dose dependent manner. The cells responded with increased proliferation rate in log phase growth, increased saturation density and change to a fibroblastic morphology. Effects shown at 2% serum in Fig. 1 for 10 ng/ml rbFGF (4.008 ± 0.006 doublings in 6 days) were found to be similar also for 100 ng/ml pituitary bFGF (4.420 ± 0.025 doublings in 6 days), 10 μg/ml ECGF (4.244 ± 0.061 doublings in 6 days) and 10 ng/ml raFGF (4.497 ± 0.186 doublings in 6 days).

The change from epithelial to fibroblastic morphology seen with testosterone [11, 32] was found also with all forms of FGF. However, only androgen and not FGF was able to maintain the characteristic transformed morphology pattern of piles of cells

interspersed with foci [11, 32]. Cells grown with TGFβ_1 had an elongated fibroblastic-like morphology but grew tightly packed together in an even monolayer. No morphological change was noted with EGF and the cells remained epithelial-like (data not shown).

Loss of steroid sensitivity in the S115 – A cells was not accompanied by loss of response to serum stimulation. The cells responded to increasing serum concentrations with increased growth rate but growth was similar at any one serum level with or without testosterone (Fig. 2). Interestingly, any response to individual growth factors in these cells was inhibitory rather than stimulatory (Fig. 2) (experiment performed twice to check for reproducibility). Furthermore, increased serum concentration reduced the effects of growth factors, in contrast to the S115 + A cells where it enhanced effects. No effect on cell morphology was noted with any growth factor in the – A cells.

Monolayer growth of S115 + A and – A cells was also studied with various combinations of growth factors at 2% serum levels (Fig. 3). For S115 + A cells, addition of bFGF and TGFβ_1 together resulted in increased growth stimulation above that seen with either growth factor alone ($P = 0.03$ vs rbFGF alone; $P = 0.001$ vs TGFβ_1 alone) but with no more than an additive effect. Further addition of EGF and insulin on top had no effect ($P = 0.23$ for rbFGF + TGFβ_1 vs rbFGF + TGFβ_1 + EGF + Ins). For S115 – A cells, however, addition of FGF and TGFβ_1 together resulted in a dramatic reduction in growth rate ($P < 0.001$ vs either rbFGF alone or TGFβ_1 alone) such that a synergistic rather than an additive effect on inhibition of cell growth was observed. Further addition of EGF and insulin on top had no effect ($P = 0.78$ for rbFGF + TGFβ_1 vs rbFGF + TGFβ_1 + EGF + Ins).

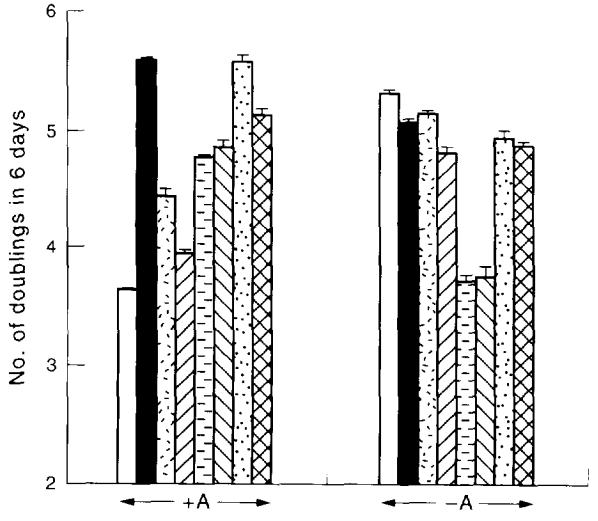

Fig. 3. Interaction of growth factors with each other and with testosterone on the growth of androgen responsive S115 + A and androgen insensitive S115 – A mouse mammary tumour cells in monolayer culture at 2% DCFCS. Cells were grown without steroid or growth factor addition (□) or with 3.5×10^{-8} M testosterone (■), 10 ng/ml rbFGF (▥), 10^{-10} M TGFβ_1 (▨), 10 ng/ml rbFGF + 10^{-10} M TGFβ_1 (▤), 10 ng/ml rbFGF + 10^{-10} M TGFβ_1 + 100 ng/ml EGF + 1 μg/ml insulin (▧), 3.5×10^{-8} M testosterone + 10 ng/ml rbFGF (▩), 3.5×10^{-8} M testosterone + 10^{-10} M TGFβ_1 (▩). Cell growth was expressed as the mean number of cell doublings in 6 days and error bars show the standard error from triplicate estimates.

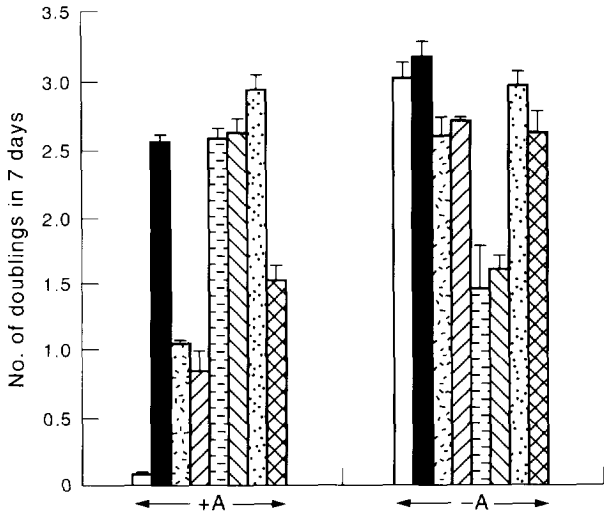

Fig. 4. Effect of growth factors on the growth of androgen responsive S115 + A and androgen insensitive S115 – A mouse mammary tumour cells in suspension culture at 2% DCFCS. Cells were grown without steroid or growth factor addition (▥) or with 3.5×10^{-8} M testosterone (■), 10 ng/ml rbFGF (▤), 10^{-10} M TGFβ (▨), 10 ng/ml rbFGF + 10^{-10} M TGFβ (□), 10 ng/ml rbFGF + 10^{-10} M TGFβ + 100 ng/ml EGF + 1 μg/ml insulin (▩), 3.5×10^{-8} M testosterone + 10 ng/ml rbFGF (■), 3.5×10^{-8} M testosterone + 10^{-10} M TGFβ (■). Cell growth was expressed as the mean number of cell doublings in 7 days and error bars show the standard error from triplicate estimates.

Growth factor regulation of anchorage-independent growth

In the absence of steroid or growth factor, S115 + A cells do not grow in suspension culture at all [11]. A full analysis of growth promoting properties of all possible single, double, treble and quadruple combinations of EGF, bFGF, TGFβ_1 and insulin was carried out on suspension growth in 2% serum and compared with the growth obtained with testosterone. On their own, only FGF ($P < 0.001$) and TGFβ_1 ($P = 0.03$) had any effect on growth and that was of growth stimulation (Fig. 4). However, when added together, FGF and TGFβ_1 had a markedly greater effect ($P = 0.001$ vs either rbFGF alone or TFGβ_1 alone), in fact so much so that growth was of the same level as with testosterone (Fig. 4). Addition of one or both of EGF or insulin had no effect on top to growth promotion ($P = 0.80$ for rbFGF + TGFβ_1 vs rbFGF + TGFβ_1 + EGF + Ins) (Fig. 4). The ability of FGF and TGFβ_1 to promote growth in suspension of these cells has been found in five separate experiments.

S115 – A cells acquire the ability to grow in suspension culture with or without testosterone [11, 27]. As found with monolayer growth, any effects of growth factors on growth of these cells in suspension was inhibitory and no longer stimulatory. Alone, only bFGF ($P = 0.07$) and TGFβ_1 ($P = 0.11$) had any effect

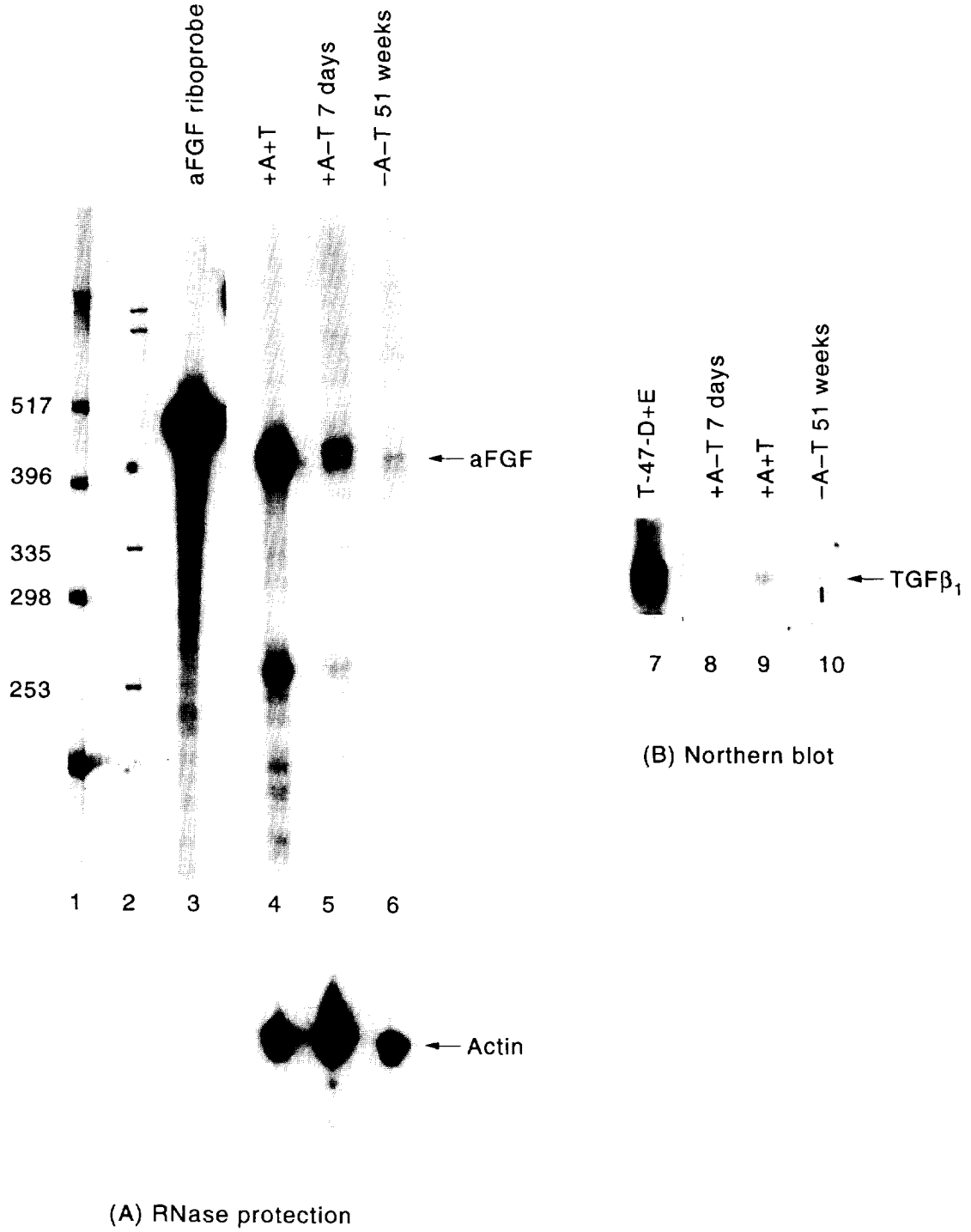

Fig. 5. Identification and steroid regulation of mRNA for aFGF (A) and TGFβ_1 (B) in S115 mouse mammary tumour cells. Whole cell RNA was isolated from monolayer cultures of androgen responsive S115 + A cells grown for 7 days in 2% DCFCS with (+A + T) (tracks 4, 9) or without (+A – T) (tracks 5, 8) 3.5×10^{-8} M testosterone and from androgen insensitive S115 – A cells grown without testosterone (–A – T) (tracks 6, 10). The –A cells were derived from +A cells by growth without testosterone for 51 weeks. RNA (10 μg tracks 4–7; 50 μg tracks 8–10) was subjected to either RNase protection analysis using antisense riboprobes for aFGF and γ-actin (A) or Northern blotting (B). Probe fragments protected in the RNase protection (A) were 480 bp for aFGF (track 3) and 75 bp for actin. Sizes of MW markers are indicated on the left-hand side (tracks 1, 2). Quantitative comparison of TGFβ_1 mRNA in T-47-D human breast cancer cells grown in the presence of 10^{-8} M oestradiol [31] and detected by Northern blotting is indicated in track 7.

and the effect of each alone was small, but when added together there was a strong inhibition of growth ($P < 0.001$) (Fig. 4).

Interaction of growth factors with testosterone on cell growth

The effects of growth factors were also studied on growth regulation of the cells in the presence of testosterone for both monolayer and suspension culture (Figs 3 and 4). For S115 + A cells growing in the presence of testosterone, bFGF showed either no effect ($P = 0.83$) (monolayer) or small stimulation ($P = 0.008$) (suspension) but TGFβ_1 was inhibitory in both monolayer ($P = 0.001$) and suspension ($P = 0.001$). Duplicate experiments confirmed effects to be reproducible. For S115 − A cells growing in the presence of testosterone, small inhibitory effects were observed in monolayer for bFGF ($P = 0.14$) and TGFβ_1 ($P = 0.01$) and in suspension for bFGF ($P = 0.29$) and TGFβ_1 ($P = 0.05$).

Steroid regulation of endogenous growth factor mRNA

Detection of growth factor mRNA in S115 cells has, to date, been limited to aFGF and TGFβ_1. RNase protection assay has revealed the presence in S115 + A cells of an aFGF mRNA which was upregulated by testosterone. Levels of this aFGF mRNA remained very low in the steroid insensitive S115 − A cells [Fig. 5(A)]. Northern blotting showed the presence also of TGFβ_1 mRNA in S115 + A cells although at low levels compared to other breast cancer cell lines [Fig. 5(B), cf. tracks 8–10 with 7]. (Growth and origin of the T-47-D cells used have been documented elsewhere [31].) Levels of TGFβ_1 mRNA were increased by testosterone in the S115 + A cells. Again, S115 − A cells had only very low levels of this TGFβ_1 mRNA [Fig. 5(B)].

DISCUSSION

We describe here growth factor gene expression and sensitivity in S115 mouse mammary tumour cells as the cells progress from a state of steroid sensitivity to insensitivity, to investigate whether steroid autonomy could result from increased endogenous growth factor production or an altered sensitivity of the cells to exogenous growth factors. The generation of steroid insensitive cells from cloned steroid sensitive antecedents [26] has enabled a direct comparison of sensitive with insensitive cells from the same clonal parent line.

Initial studies describe the changes in sensitivity of the cells to exogenous growth factors. In terms of sensitivity to serum, alterations were only observed at high serum levels. At low serum levels (0.5–2%) unresponsive cells grew at the same rate as androgen-stimulated responsive cells, but at higher serum levels (10%) the unresponsive cells acquired an ability to grow faster. It remains in question at to whether this reflects development of independence from an inhibitory element in serum or increased sensitivity to growth factors present at low concentration in the serum.

Steroid responsive S115 + A cells showed a broadly similar sensitivity to individual growth factors as reported for SC3 cells [16, 17, 33] in that cell growth was stimulated by various forms of FGF and by TGFβ in both anchorage dependent and independent culture. This demonstrates that results are not specific to one clone of cells within one laboratory but may be a feature more general to the S115 tumour. During progression to autonomy, however, there was a general alteration in growth factor sensitivity such that growth factors became inhibitory to the unresponsive cells in all cultures, with a particularly striking synergistic action between FGF and TGFβ. Interestingly, the observed effects were influenced reciprocally by serum in that growth factor stimulation of the responsive cells was dependent on high serum levels but inhibitory action on the unresponsive cells was greater at lower serum levels. Such alterations in sensitivity could offer one explanation for inter-laboratory discrepancies concerning stimulatory and inhibitory effects of serum growth factors in breast cancer cells *in vitro* [34]. Altered response to FGF has been reported between androgen sensitive and insensitive prostate cancer cell lines [7]. Reversal of TGFβ sensitivity has been described in SC3 cells during short-term alterations to androgen environment [33]. TGFβ alone was shown to be stimulatory to responsive SC3 cells but became inhibitory in the short-term presence of testosterone. Molecular studies showed that the altered response in the presence of testosterone resulted from the ability of TGFβ to inhibit secretion and hence growth stimulation by the androgen-induced growth factor AIGF [33]. Interestingly, inhibitory effects of TGFβ alone on cell growth *in vitro* have also been recorded in prostate cancer epithelial cells [35]. Clearly, molecular mechanisms during progression to autonomy also need to be elucidated.

RNase protection analysis revealed the presence of a genuine aFGF mRNA in steroid responsive S115 + A cells which was upregulated by androgen. The extent to which this RNA is involved in the growth response remains in question. The AIGF of SC3 cells may well be involved in growth at least to some extent since FGF antibodies can reduce testosterone stimulation of growth [13]. However, it is unlikely that FGF alone will be sufficient to explain all the growth properties of androgen [36]. In these experiments, exogenous FGF alone was not sufficient to maintain the fully transformed morphological phenotype to the same extent as androgen. Also, FGF could enhance only DNA synthesis and not total cell yield (saturation density) in SC3 cells, unlike androgen which can do both [36]. In addition, antibodies (R&D systems) which block biological action of bFGF, aFGF or TGFβ, when tested individually, had only partially inhibitory actions on

androgen regulation of S115 cell growth (data unpublished) or SC3 cells [17]. Furthermore, FGF had only a small effect compared to androgen in promoting growth in suspension culture, one of the parameters most closely linked to tumorigenesis *in vivo* [37, 38]. In this respect, it is interesting that the combination of FGF with TGFβ_1 did promote growth in suspension to the same degree as androgen. It could be that the androgen responses are mediated by more than one growth factor, and TGFβ_1 mRNA was indeed also regulated by androgen in the S115 + A cells.

The molecular mechanism of androgen regulation of the aFGF gene expression in these cells remains to be elucidated. The promoter organization of the aFGF gene appears to be very complex allowing the use of four different promoters to generate four different transcripts termed 1.A [39], 1.B [40], 1.C [41] and 1.D [41]. Recent work has shown androgen regulation of the 1.C aFGF mRNA in a hamster ductus deferens derived smooth muscle tumour cell line DDT-1 [6] and the human prostatic cancer cell line LNCaP [42] and furthermore, androgen regulatory sequences have been identified in the 5′ noncoding region by CAT reporter gene assays in the DDT-1 cells [43] and by DNA sequencing of the human 1.C promoter [41]. At this time, it remains a possibility that 1.C mRNA is restricted to certain cell types within the prostate [41] and has been suggested to play a role in androgen-induced tumorigenesis [41]. If the aFGF mRNA here is also 1.C then this would provide an example of synthesis outside the prostate in breast epithelial cancer cells. If it is not 1.C, then it would provide an example of a novel androgen-regulated aFGF mRNA.

Whatever the molecular mechanism and physiological role of aFGF in growth regulation of the steroid sensitive tumour cells, there was no upregulation of aFGF mRNA in the unresponsive cells. This demonstrates that loss of response in these cells does not occur simply by constitutive upregulation of growth factors which were previously androgen-regulated. Furthermore, if growth factors have, in general, an inhibitory effect on the steroid insensitive cells, it is difficult to postulate how simple upregulation of endogenous growth factor production could be involved in the upregulation of growth seen in the loss of response mechanism. If growth factor pathways are involved, upregulation of receptors would seem now to be a more likely mechanism. Overexpression of receptors for EGF [44] or FGF [45] have already been implicated in downregulation of breast cancer cell growth by ligand. At a clinical level, gross inhibition of steroid insensitive cells by combinations of growth factors could provide a new way for reducing tumour cell growth in certain cases where endocrine therapy fails.

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