citrate counteracts the antitumor effects of cytotoxic drugs *in vitro*. *J Clin Oncol* 1985, 3, 1672–1677.
9. Osborne KC, Kitten L, Arteaga CL. Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens. *J Clin Oncol* 1989, 7, 710–717.
10. Scambia G, Ranelletti FO, Panici PB, *et al.* Synergistic anti proliferative activity of tamoxifen and cisplatin on primary ovarian tumours. *Eur J Cancer* 1992, 11, 1885–1889.
11. Cocconi G, Bella M, Calabresi F, *et al.* Treatment of metastatic malignant melanoma with dacarbazine plus tamoxifen. *N Engl J Med* 1992, 327, 516–523.
12. Calabresi F, Aapro M, Becquart D, *et al.* Multicenter phase II trial of the single agent fotemustine in patients with advanced malignant melanoma. *Ann Oncol* 1991, 2, 377–378.
13. Sutherland RL, Hall RE, Pang GYN, Musgrove EA, Clark CL. Effect of medroxyprogesterone acetate on proliferation and cell kinetics of human mammary carcinoma cells. *Cancer Res* 1988, 48, 5084–5091.
14. Scatchard G. The attractions of proteins for small molecules and ions. *Ann NY Acad Sci USA* 1949, 51, 660–672.
15. Lokiec F, Beerblock K, Deloffre P, Lucas C, Bizzari JP. Clinical pharmacokinetics study of fotemustine in different tumor types. *Bull Cancer* 1989, 76, 1063–1069.
16. Etienne MC, Milano G, Fischel JL, *et al.* Tamoxifen metabolism: pharmacokinetic and *in vitro* study. *Br J Cancer* 1989, 60, 30–35.
17. Carmichael J, De Graff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semi-automated colorimetric assay in assessment of chemosensitivity testing. *Cancer Res* 1987, 47, 936–942.
18. McClay EF, Mastrangelo MJ, Sprancho ID, Bellet RE, Berd D. The importance of tamoxifen to a cisplatin-containing regimen in the treatment of metastatic melanoma. *Cancer* 1989, 63, 1292–1295.
19. McClay EF, Albright KD, Jones JA, *et al.* Modulation of cisplatin resistance in human malignant melanoma cells. *Cancer Res* 1992, 52, 6790–6796.
20. Zava DT, Goldhirsh A. Estrogen receptor in malignant melanoma: fact or artefact? *Eur J Cancer Clin Oncol* 1983, 19, 1151–1159.
21. Etienne MC, Fischel JL, Milano G, *et al.* Steroid receptors in human osteoblast-like cells. *Eur J Cancer* 1990, 26, 807–810.
22. Meyn RE and Murray D. Cell cycle effects of alkylating agents. *Pharmac Ther* 1984, 24, 147–163.
23. Love RR. Tamoxifen therapy in primary breast cancer: biology, efficacy, and side effects. *J Clin Oncol* 1989, 7, 803–815.
24. Clarke R, Van der Berg HW, Murphy RF. Reduction of the membrane fluidity of human breast cancer cells by tamoxifen and 17 β-estradiol. *J Natl Cancer Inst* 1990, 82, 1702–1705.
25. Lien EA, Solheim E, Ueland PM. Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. *Cancer Res* 1991, 51, 4837–4844.

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Acute Effects of Growth Factors on T-47D Breast Cancer Cell Cycle Progression

Elizabeth A. Musgrove and Robert L. Sutherland

Growth factors play a major role in the control of human breast cancer cell proliferation but their acute effects on cell cycle progression have not been well studied in these cells. T-47D cells, growth-inhibited by serum deprivation, were induced to re-enter the cell cycle in a concentration- and time-dependent manner by addition of insulin, insulin-like growth factor (IGF)-I, epidermal growth factor (EGF), transforming growth factor α (TGFα) or basic fibroblast growth factor (bFGF). After a lag of ~10 h semi-synchronous entry into S phase was observed. The relative potencies of maximal concentrations of growth factors were in the order: insulin ≈ IGF-I ≈ bFGF > TGFα > EGF, identifying bFGF as among the most potent mitogens for these cells. Insulin or IGF-I alone resulted in growth rates comparable with those observed in fetal calf serum. These data demonstrate that single growth factors can induce a significant proportion of T-47D cells to traverse the cell cycle. The kinetics for entry into S phase were similar, indicating that the basis of differential sensitivity to the growth factors tested was the proportion of cells that responded and ultimately entered S phase.
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INTRODUCTION

BREAST CANCER cell lines respond not only to steroid hormones, but also to a wide range of peptide hormones and growth factors [1–3] and thus provide an experimental model in which the actions of a variety of mitogens can be studied. Members of the epidermal growth factor (EGF)/transforming growth factor α (TGFα) and insulin/insulin-like growth factor-I (IGF-I) family have been widely investigated using these cells, a reflection of the major role these growth factors are thought to play in the overall regulation of proliferation of breast cancer cells. Indeed, some of the earliest studies using breast cancer cells in tissue culture documented growth stimulation by insulin and EGF [4, 5]. However, there is evidence that other, less well-studied growth factors are important in aspects of normal breast and mammary gland development, as well as in the initiation or progression of tumours. For example, mammary epithelial cells respond to several fibroblast growth factor homologues [6–8]. Proliferation of MCF-7 and T-47D human breast cancer cells is also stimulated by both acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) [9, 10], although other studies have found no effect, even in the absence of other mitogens [11].

Despite the diverse growth factors to which responses have been recorded, relatively few studies have directly compared the effects of a range of growth factors on breast cancer cell proliferation. It is clear, however, that the degree of response differs between growth factors. For example, in one publication EGF, TGFα and bFGF promoted fewer cell population doublings of MCF-7 and T-47D cells than did insulin or IGF-I [9]. Similarly, another study using MCF-7 cells found insulin to be

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considerably more potent than a range of other growth factors including EGF and bFGF [11]. The basis for this differential response has not been identified, but may include differences in the growth rate of responsive cells or in the proportion of cells which respond. The majority of studies have primarily been concerned with long-term effects, using increases in the relative cell number as an end-point. Few studies have addressed in any detail the changes in cell cycle kinetics accompanying changes in growth rate, particularly in defined culture conditions. However, in other cell types, particularly fibroblasts, insight into the contribution of growth factors to the regulation of proliferation, for example, the concepts of 'competence' and 'progression' growth factors, has been gained by comparison of acute effects on cell cycle progression. Such a model has not been demonstrated for epithelial cells, but establishing the cell cycle kinetic basis for differing responses to growth factors in breast cancer cells may allow identification of any common mechanisms. Such data may also provide a starting point for the eventual identification of growth regulatory mechanisms unique to breast cancer cells.

To define the cell cycle kinetic basis for the differential response to individual growth factors, the aim of this study was to compare the acute effects of polypeptide mitogens from three major families (insulin/IGF-I, EGF/TGFα, bFGF) on T-47D breast cancer cells. The exogenous growth factors normally present in fetal calf serum (FCS) and the usually high proliferation rates of cells maintained in the presence of FCS are both likely to decrease the ability to detect responses to additional growth factors. In addition, altered sensitivity to components of the extracellular environment may indirectly modulate the growth rate in treated cultures. The use of a hormonally-defined model system may be necessary to distinguish between truly growth regulatory actions and these indirect effects. Therefore, to allow the greatest sensitivity for detection of cell cycle progression induced by mitogenic growth factors, the effects of individual growth factors were tested using cells that had been growth-inhibited by serum deprivation and were then maintained in serum-free medium.

MATERIALS AND METHODS

Reagents

Following the supplier's recommendations, growth factors were dissolved as follows: porcine insulin (generously donated by John Miller, CSL-Novo, North Rocks, New South Wales, Australia) ≥95% pure, 10 mg/ml in 10 mmol/l HCl; human recombinant IGF-I (Boehringer Mannheim, Castle Hill, New South Wales, Australia), 1.0 μmol/l in 1 mmol/l HCl/27 μmol/l human transferrin; murine EGF (Collaborative Research, Bedford, Massachusetts, U.S.A.), 100 μmol/l in distilled water; human recombinant TGFα (Bachem Feinchemikalien, Bubendorf, Switzerland), 10 μmol/l in distilled water; human recombinant bFGF (generously donated by Dr A. Protter, Pacific Biotechnology, Sydney, New South Wales, Australia), 0.6 μmol/l in 30 μmol/l human transferrin. All growth factors were 98–99% pure. Insulin was filter-sterilised; other growth factors were used without filtration. These stock solutions were stored in aliquots at −20°C and were diluted further on the day of use if necessary, in general to give a 1000-fold concentrated solution. Other reagents were obtained from standard commercial suppliers, except as noted in the text.

Cell culture

Stock cultures of T-47D human breast cancer cells, obtained at passage 74 from E.G. and G. Mason Research Institute (Worcester, Massachusetts, U.S.A.) were maintained as previously described but without antibiotics, in RPMI 1640 medium supplemented with 10 μg/ml porcine insulin (Actrapid, CSL-Novo, North Rocks, New South Wales, Australia) and 10% FCS [12]. The medium used for experiments consisted of phenol red-free RPMI 1640, containing *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (20 mmol/l), sodium bicarbonate (14 mmol/l), L-glutamine (6 mmol/l) and 20 μg/ml gentamycin. Serum-free medium also contained 300 nmol/l human transferrin (Sigma Chemical Co., St Louis, Missouri, U.S.A.); alternatively, the base medium was supplemented with filter-sterilised charcoal-treated FCS [13]. Steroids present in FCS are adsorbed by activated charcoal and this treatment depletes >99% of added trace 17β-[^{3}H] oestradiol [13].

Prior to each experiment cells taken from stock cultures were passaged for 5–7 days, in medium containing 10% charcoal-treated FCS, with two changes of medium at 1–3-day intervals. These cells were replated into replicate flasks (1 or 1.5 × 10^5 cells/25 cm^2 flask) in 5 ml medium containing 15% charcoal-treated FCS. On 2 successive days thereafter the medium was replaced with fresh serum-free medium, which was then not further replaced during the course of the experiment. Each experiment commenced 3–5 days after the final medium change, with the addition of growth factor directly into the medium. Equal volumes of vehicle were added to control cultures. At the indicated times thereafter flasks were harvested with 0.05% trypsin–0.02% EDTA in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (1.5 mmol/l KH_2PO_4, 8.1 mmol/l Na_2HPO_4, 2.7 mmol/l KCl, 140 mmol/l NaCl).

DNA flow cytometry

Harvested cells were resuspended in tissue culture medium containing 5–10% FCS and, after cell counting using a haemocytometer under phase-contrast microscopy where appropriate, stained for later DNA analysis by the addition of 40 μg/ml ethidium bromide (Sigma) and 12.5 μg/ml mithramycin (Pfizer, West Ryde, New South Wales, Australia) in the presence of 0.2% v/v Triton X-100. DNA histograms were obtained using a FACStar flow cytometer (Becton Dickinson Immunocytochemistry Systems, Mountain View, California, U.S.A.) and the cell cycle phase distribution estimated using the manufacturer's DNA analysis software. Each histogram contained 30 000 events, and typically had a coefficient of variation for the G_1 peak of 2–3%.

Statistics

Regression lines were fitted to percentage (%) S phase data from detailed time-course experiments using StatView 512$^+$ software. The equations were then used to calculate the intercept between the regression line for each treatment group and a horizontal line through the mean value for control cultures. This intercept was taken to be the time of entry into S phase. The standard error (S.E.) of the fitted slope was used to estimate the S.E in the time of entry into S phase. The statistical significance of differences in the time or rate of entry into S phase was evaluated using the Student's *t*-test.

RESULTS

Following steroid depletion and replating into medium containing charcoal-treated FCS, two successive changes of serum-free medium served to additionally deplete the cells of growth factors present in the serum-containing medium. After these pretreatments, T-47D cells remained viable and attached to the

surface of the tissue culture flask, and had similar morphology to that of cells maintained in 10% FCS. However, cell numbers remained static for a lag period of 1–3 days after the final medium change and thereafter increased slowly. The reduction in growth rate in serum-free medium was accompanied by a marked decrease in the proportion of cells in S phase, to less than half that observed in cultures maintained in the presence of FCS. The proportion of cells with G_0/G_1 DNA content correspondingly rose. These observations suggest that the experimental protocol used to deplete mitogens from the culture medium leads to growth arrest for the majority of T-47D cells.

Effects of insulin or IGF-I

Initial experiments used high concentrations of insulin (1.7 μmol/l) or IGF-I (10 nmol/l) to determine the time-course of entry into S phase for the stimulated cells. The data obtained for insulin and IGF-I were identical in both timing and magnitude (Fig. 1a). By 12 h, the proportion of cells in S phase had begun to increase, and reached its maximum at 18–24 h. The proportion of cells in G_1 phase decreased simultaneously, while the $\%G_2$ + M phase increased after a delay (data not shown). Thus the changes in cell cycle phase distribution were consistent with semi-synchronous entry into S phase, and the apparently cyclic variation at later times (Fig. 1a) suggests that at least one further round of semi-synchronous replication occurred. The addition of FCS to a final concentration of 10% resulted in changes in cell cycle phase distribution which were of larger magnitude but similar time-course to that observed after insulin addition (Fig. 1a).

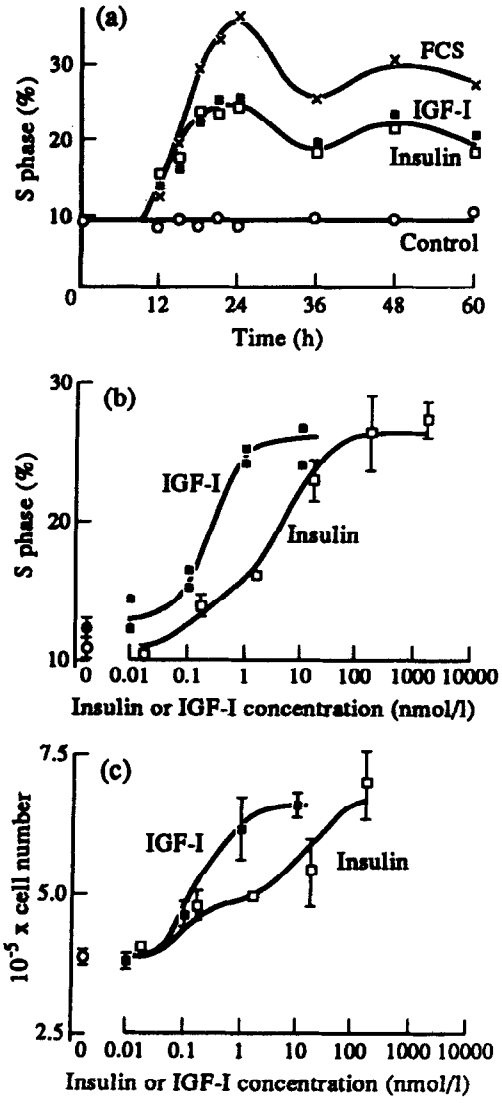

Fig. 1. Time and concentration dependence of increases in S phase fraction and cell number following treatment of T-47D cells with insulin or IGF-I. (a) T-47D cells cultured in serum-free medium were treated with insulin (1.7 μmol/l, □), IGF-I (10 nmol/l, ■), FCS (10%, ×) or vehicle (○,●). (b) S phase fraction after 21 or 24 h treatment. (c) Cell counts after 96 h treatment (mean ± S.E.M. of the cell number/25 cm² flask).

The concentration-dependence of the response to insulin or IGF-I was determined by flow cytometric DNA analysis of cultures harvested after 24 h treatment, when the S phase fraction was at its peak. For IGF-I-treated cells some response was evident at 0.1 nmol/l and the maximum response, an approximately 2-fold increase in the S phase fraction, was observed at 1 nmol/l (Fig. 1b). Increases in cell numbers were correspondingly apparent after 96 h treatment (Fig. 1c). Insulin was as effective as a maximum concentration of IGF-I, and increases in cell number at 96 h were in general as expected from S phase responses at 24 h. The effects of insulin increased over a wide concentration range such that responses in either S phase or cell number were first detectable at a concentration of 0.17 nmol/l and increased with increasing insulin concentrations up to 0.17 μmol/l. An insulin concentration of 1.7 μmol/l appeared to be no more effective in increasing S phase than 0.17 μmol/l.

Effects of EGF or TGFα

Treatment of T-47D cells with 10 nmol/l human recombinant TGFα or 10 nmol/l murine EGF resulted in changes in cell cycle phase distribution temporally similar to those observed upon insulin or IGF-I treatment in the same experimental design, such that stimulated cells entered S phase semi-synchronously at approximately 12 h. Neither growth factor was as effective as insulin or IGF-I, but TGFα caused a marked increase in %S phase by 18 h, in contrast with the modest response to EGF (Fig. 2a). The proportion of cells in S phase remained elevated above control values for at least 60 h. Examination of the increase in %S phase at 18 h resulting from a range of TGFα concentrations showed TGFα to be potent, such that the effects were half-maximal at 0.01–0.02 nmol/l, and near-maximal at 0.1 nmol/l (Fig. 2b). EGF was consistently much less effective than TGFα, and responses were difficult to detect in some experiments; Fig. 2b shows small responses over a wide range of concentrations (0.01–10 nmol/l), with no clear concentration dependence. This result does not appear to reflect the use of murine EGF or the particular preparation of EGF used, since human recombinant EGF was also poorly mitogenic (data not shown).

Effects of bFGF

Human recombinant bFGF (0.6–1.2 nmol/l) was as effective as insulin or IGF-I in stimulating T-47D cell cycle progression in serum-free medium (compare Figs 1a and 3a). The %S phase increased with 14 h exposure and reached a maximum by approximately 18 h (Fig. 3a), a similar time-course to that observed for cells treated with other growth factors. The S phase fraction remained elevated for at least 48 h. Studies of the response to different concentrations showed that bFGF was not only effective but also potent: concentrations as low as 30 pmol/l led to increases in %S phase similar to those observed with

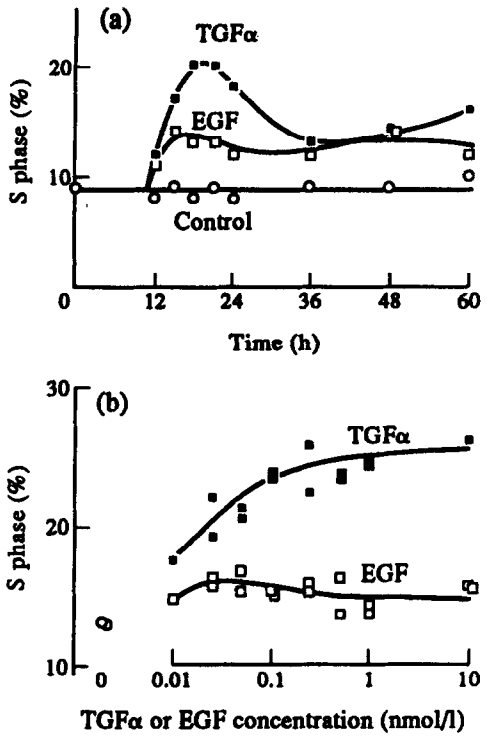

Fig. 2. Time and concentration dependence of increases in S phase fraction following treatment of T-47D cells with TGFα or EGF. (a) T-47D cells cultured in serum-free medium were treated with TGFα (10 nmol/l, ■), EGF (10 nmol/l, □), or vehicle (○). (b) S phase fraction after 18 h treatment.

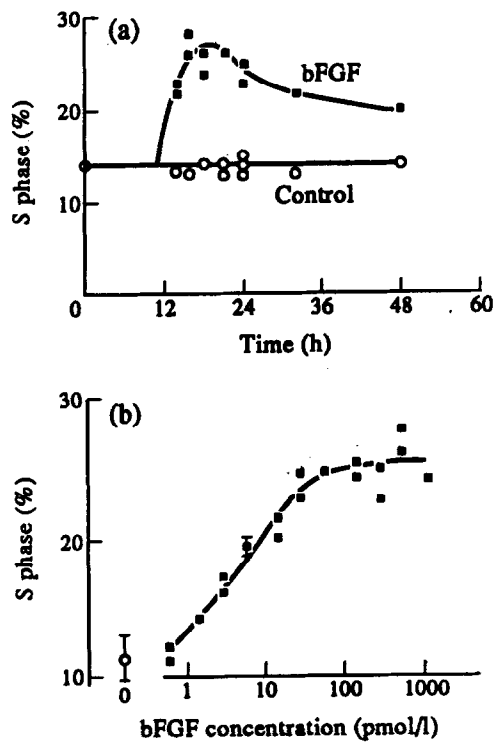

Fig. 3. Time and concentration dependence of increases in S phase fraction following treatment of T-47D cells with bFGF. (a) T-47D cells cultured in serum-free medium were treated with bFGF (0.6 or 1.2 nmol/l, ■) or vehicle (○). (b) S phase fraction after 21 h treatment. Where error bars are shown, points denote mean ± S.E.M. of four or five histograms.

1.2 nmol/l, and the effects were half-maximal at concentrations of approximately 5 pmol/l or lower (Fig. 3b).

Effects on proliferation rate

For the growth factors examined, increases in cell number with long-term treatments in general reflected the same order of effectiveness as did the changes in cell cycle phase distribution. A representative experiment is shown in Fig. 4. Insulin and IGF-I led to sustained rapid growth, as did FCS, with doubling times of 1.8 days for FCS and 1.9 days for pooled insulin and IGF-I data in this experiment (calculated from cell counts at 60 and 130 h). In contrast, the doubling time of cells in unsupplemented serum-free medium was 6 days. Cells treated with TGFα initially increased in number at a rate comparable with that upon insulin or IGF-I stimulation, but with longer exposure the growth rate fell (Fig. 4). Treatment with bFGF led to growth curves of similar shape to those obtained after TGFα treatment (data not shown). Within 48 h the cell number in the presence of bFGF had increased by 80% relative to parallel cultures in unsupplemented serum-free medium. However, this increased growth rate was not sustained upon longer treatment. These data confirm that cells which enter S phase after stimulation by these growth factors subsequently complete the cell cycle and divide.

Relative potencies and kinetics of entry into S phase

To compare directly the potencies of growth factors from each of the classes investigated, the maximum proportion of cells in S phase was determined in parallel cultures treated with insulin (1.7 μmol/l), IGF-I (10 nmol/l), TGFα (10 nmol/l) or bFGF (0.6 nmol/l). These concentrations were maximally effective in the experiments presented in Figs 1–3. Insulin, IGF-I and bFGF induced increases in %S phase of similar magnitude, approximately 60% of that induced by FCS, while the response to TGFα was consistently more modest, ~70% of the response to insulin, and that of EGF less than half that of insulin (Fig. 5).

Differences in the maximum %S phase could result from differing proportions of responsive cells, or differences in the

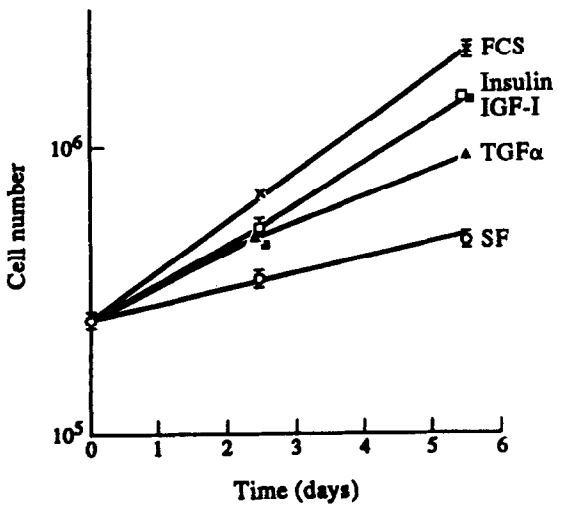

Fig. 4. Increase in cell number of T-47D cells in the presence or absence of FCS, insulin, IGF-I or TGFα. T-47D cells cultured in serum-free (SF) medium were treated with FCS (10%, ×), insulin (1.7 μmol/l, □), IGF-I (10 nmol, ■), TGFα (10 nmol/l, ▲) or vehicle (SF, ○). Points represent the mean cell number/25 cm² flask; error bars represent the range of duplicate cell counts.

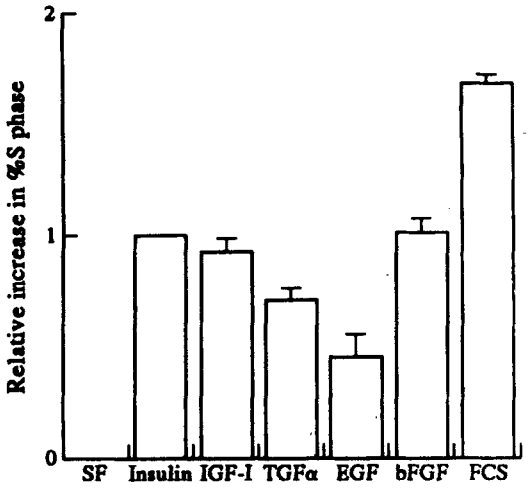

Fig. 5. Relative effects of insulin, IGF-1, TGFα, EGF, bFGF and FCS on S phase fraction of T-47D cells. Either 1.7 μmol/l insulin, 10 nmol/l IGF-I, 10 nmol/l TGFα, 10 nmol/l EGF, 0.6 nmol/l bFGF or FCS (10%) was added to T-47D cells cultured in serum-free medium. Increases in %S phase after 16–24 h treatment have been calculated by subtraction of the %S phase of cultures maintained in serum-free medium alone (SF), then normalised to the value obtained for insulin. Data have been pooled from three to five separate experiments. Error bars indicate S.E.M.

kinetics of entry into S phase. The time-courses of changes in %S phase were broadly similar for the growth factors examined (Figs 2a, 3a and 4a), suggesting that the time of entry into S phase was similar in each case. To directly compare the time and rate at which cells stimulated by individual growth factors entered S phase, detailed time-course studies were performed, using parallel cultures treated with maximally effective concentrations of each growth factor (as above), for 12–17 h. The data were plotted using a logarithmic scale for %S phase and regression analysis used to determine the rates of entry into S phase (i.e. the slope of the regression line) and the time of entry into S phase. Neither the rates of entry into S phase nor the times at which cells began to enter S phase were significantly different for any of the treatment groups (Table 1).

DISCUSSION

The model system described here provides a sensitive means of investigating the effects of mitogens on breast cancer cell lines. The growth arrest of cells maintained in serum-free medium and the low growth factor concentrations required to elicit proliferation argue against the retention of significant concentrations of mitogens following repeated changes of serum-free medium. In serum-free medium, growth factors from each of the three growth factor families tested (insulin/IGF-I, EGF/TGFα and bFGF) were capable of stimulating T-47D cell proliferation such that a semi-synchronous cohort of cells entered S phase, approximately 10 h after the beginning of treatment. However, no individual growth factor was as effective as FCS. The growth factors can be ranked in order of increasing ability to acutely stimulate cell cycle progression: EGF < TGFα < insulin = IGF-I ≈ bFGF < FCS. Each of the growth factors which elicited substantial responses in these experiments (excluding insulin) was half-maximally effective at a concentration approximately 10-fold lower than the affinity of the growth factor for its cognate receptor, i.e. at a concentration where few of the receptor sites would be occupied. The data are summarised in Table 2, shown for comparison with data from a publication of Karey and Sirbasku [9] which appears to be the only study to investigate such a range of growth factors under similar conditions using T-47D cells. Despite the differing endpoints, i.e. relative cell number upon long-term exposure compared with %S phase on acute exposure, there is often qualitative agreement upon the relative effectiveness of the growth factors. For both acute and long-term effects on cell proliferation (see Table 2), EGF and TGFα were less effective than insulin or IGF-I. In short-term experiments in this study bFGF was as effective as insulin or IGF-I, while in the study of Karey and Sirbasku [9], bFGF induced only modest relative increases in cell number. Both TGFα and bFGF were more potent in increasing the S phase fraction than in increasing the relative cell number: in each case the concentration found to be half-maximal by Karey and Sirbasku [9] is approximately the concentration which was maximally effective here. These data suggest that degradation of bFGF and TGFα, or loss of sensitivity to them, may reduce their long-term effectiveness on T-47D cells. Accelerated degradation under some culture conditions may thus be an explanation for the previously reported inability to detect responses to FGFs in some studies.

Insulin is often regarded as potentiating other growth factors by improving cell viability and increasing the basal metabolic rate, but can be mitogenic at high concentrations [14, 15]. In contrast, IGF-I *in vitro* and *in vivo* has metabolic effects only at high concentrations but has effects on cell proliferation at concentrations of 1 nmol/l or less [14, 15]. These data have been

Table 1. Time and rate of S phase entry after growth factor stimulation

Growth factor	r^{2*}	Rate† (arbitrary units)	Time‡ (h)
Insulin (1.7 μmol/l)	0.988	0.055±0.003	10.1±0.6
IGF-1 (10 nmol/l)	0.887	0.043±0.008	9.8±1.8
TGFα (10 nmol/l)	0.933	0.057±0.008	11.5±1.6
bFGF (0.6 nmol/l)	0.963	0.053±0.005	10.3±1.0
FCS (10%)	0.955	0.064±0.010	9.7±1.5

*Correlation coefficient for regression line. †Slope of regression line, i.e. rate of entry into S phase. ‡Time of entry into S phase, calculated from equation of regression line.

Table 2. Summary of effects of growth factors on T-47D cell proliferation in serum-free medium

	K_d* (nmol/l)	ED_{50}† (% S phase)‡ (nmol/l)	Relative %S phase§	ED_{50}† (growth)‖ (nmol/l)	Number of doublings‖
Insulin	2.0	3.5	1.00	1.7	2.4–3.0
IGF-I	1.5	0.2	0.93	0.22	2.4–3.0
TGFα	0.3	0.02	0.71	0.10	1.5
EGF	0.1		0.45	0.006	1.1
bFGF	0.05	0.005	1.02	0.025	1.4

*Apparent dissociation constant for binding to cognate receptor [10, 14, 15, 28]. †Concentration required for half-maximal effect. ‡Estimated from data presented in Figs 1b, 2b and 3b. §Presented in Fig. 4. ‖From data presented in [9]. Number of doublings indicates the maximum increase in cell number relative to that on day 0.

interpreted as evidence that metabolic effects are mediated by the insulin receptor while effects on cellular replication are mediated by the IGF-I receptor. However, mitogenic effects have now been demonstrated in normally unresponsive cells transfected with either insulin or IGF-I receptor cDNAs [16, 17]. In breast cancer cells the mitogenic activities of insulin have been most consistently observed at concentrations of $\geq$ 200 nmol/l, and have thus usually been attributed to actions mediated by binding to the IGF-I receptor. However, some studies have reported responses to much lower insulin concentrations [9, 11] and antibody blockade of the IGF-I receptor does not prevent insulin stimulation of proliferation [18]. In the experiments reported here, the response to insulin increases gradually over a 1000-fold increase in insulin concentration, consistent with binding to several sites of differing affinity. The maximal response was identical to that of IGF-I, in both acute and long-term effects on cell proliferation. These data support the conclusion that insulin stimulates breast cancer cell proliferation via its own receptor at low concentrations, but acts at least in part through binding to the IGF-I receptor at high concentrations. An additional possibility is suggested by the observation that the IGF-I and insulin receptors can form complexes in which insulin binding indirectly leads to IGF-I receptor phosphorylation [14, and references therein]. Such activation of the receptor tyrosine kinase tightly regulates receptor signalling. Thus, cross-phosphorylation may serve as an amplification step in signal transduction, particularly since the IGF-I receptor cytoplasmic domain (which includes the tyrosine kinase domain) has been shown to be more potent in activating thymidine incorporation than the corresponding domain of the insulin receptor [19].

In breast cancer cells proliferative responses to TGFα and EGF have not been exhaustively compared. In this study, EGF was consistently less effective than TGFα in stimulating entry of cells into S phase, while in another study EGF stimulated fewer population doublings than TGFα [19] (see Table 2). The divergent response appears to have some cell-type specificity, since either EGF or TGFα stimulated approximately three doublings of MCF-7 cells [9]. Although the biological response elicited by these growth factors is qualitatively similar in most experimental models, there are often quantitative differences between the responses to TGFα and EGF [20, 21, and references therein]. These differences are not due simply to differences in affinity for the receptor, but appear to result from differential processing of ligand–receptor complexes [21]. In contrast with EGF, TGFα does not fully downregulate the EGF receptor, allowing continuous availability of the receptor [21]. The resulting more sustained signal may explain the greater effectiveness of TGFα in longer term responses including mitogenesis.

The profound mitogenic response of T-47D cells to bFGF demonstrated in this study, raises the possibility that FGFs may be mitogens for normal or neoplastic breast cells *in vivo*. Indeed, mammary hyperplasia has been reported in transgenic mice expressing *int-2*, a member of the FGF family of peptides [22]. Some, but not all, mammary carcinoma cell lines produce bFGF [10, 23, 24]. However, bFGF is ubiquitously present *in vivo* within the basement membrane. Proteases secreted by breast cancer cells might release biologically active bFGF from sites on the extracellular matrix, and indirect evidence supports the possibility that cathepsin D might function in this role [10].

The time taken to reach S phase for T-47D cells stimulated by FCS was a minimum of approximately 10 h (Table 1) and on average approximately 14 h. Similar estimates for the G_1 phase duration of T-47D cells can be obtained from the cell cycle phase distribution of rapidly proliferating populations: T-47D cells growing at apparently maximal rates, with a population doubling time of 22 h, have a steady-state G_0/G_1 phase percentage of 62.5% [12]. This allows calculation of a mean G_1 phase duration of ~14 h. This figure is in good agreement with the mean time to reach S phase after FCS treatment and suggests that in unsupplemented serum-free medium, T-47D cells are arrested near the beginning of G_1 phase and rapidly re-enter the cell cycle upon mitogenic stimulation. Since the kinetics of entry into S phase are similar for each of the growth factors tested, differences in the maximum stimulation of entry into S phase result from differences in the proportion of sensitive cells. Although a similar rate of entry into S phase was observed for all growth factors tested, this does not represent the maximum rate achievable: the rate of S phase entry of T-47D cells proliferating in insulin-supplemented serum-free medium is increased upon treatment with synthetic progestins [25].

T-47D cells, like other oestrogen receptor-positive breast cancer cell lines, express low numbers of EGF receptors [26], which may explain the relatively poor mitogenic response to TGFα or EGF found in this and other studies [9, 11]. Alternatively, although the respective receptors are all tyrosine kinases, it is likely that there is some specificity in the receptor substrates. In support of this view, insulin stimulation does not preclude additional responses to EGF or TGFα, since these growth factors stimulate a similar number of cells to progress into S phase in cultures proliferating in insulin-supplemented serum-free medium or maintained in unsupplemented serum-free medium (unpublished data). Availability of receptor substrates or more distal components of the signalling pathways downstream of the EGF receptor may limit the mitogenic capacity of the EGF receptor in these cells. In either case, the different mitogenic responses to TGFα, insulin, IGF-I and bFGF would be expected to be reflected in different responses of genes or gene products controlling cell cycle progression. The recent identification of growth factor-responsive cyclin genes, postulated to have a role in controlling progress through G_1 phase, suggests new candidate genes for testing this hypothesis [27].

1. Wakeling AE. Mechanisms of growth regulation of human breast cancer. *Baillieres Clin Endocrinol Metab* 1990, **4**, 51–66.
2. Musgrove EA, Sutherland RL. Steroids, growth factors and cell cycle controls in breast cancer. In Lippman ME, Dickson RB, eds. *Regulatory Mechanisms in Breast Cancer*. Boston, Kluwer Academic Publishers, 1991, 305–331.
3. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr Rev* 1987, **8**, 29–43.
4. Turkington RW. Stimulation of mammary carcinoma cell proliferation by epidermal growth factor *in vitro*. *Cancer Res* 1969, **29**, 1457–1458.
5. Osborne CK, Bolan G, Monaco ME, Lippman ME. Hormone responsive human breast cancer in long-term tissue culture: effect of insulin. *Proc Natl Acad Sci USA* 1976, **73**, 4536–4540.
6. Takahashi K, Suzuki K, Kawahara S, Ono T. Growth stimulation of human breast epithelial cells by basic fibroblast growth factor in serum-free medium. *Int J Cancer* 1989, **43**, 870–874.
7. Dixon M, Deed R, Acland P, *et al.* Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. *Mol Cell Biol* 1989, **9**, 4869–4902.
8. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc Natl Acad Sci USA* 1989, **86**, 802–806.
9. Karey KP. Sirbasku DA. Differential responsiveness of human

breast cancer cell lines MCF-7 and T47D to growth factors and 17 β-estradiol. *Cancer Res* 1988, **48**, 4083–4092.
10. Briozzo P, Badet J, Capony F, *et al.* MCF7 mammary carcinoma cells respond to bFGF and internalize it following its release from extracellular matrix: a permissive role of cathepsin D. *Exp Cell Res* 1991, **194**, 252–259.
11. van der Burg B, Rutteman GR, Blankenstein MA, de Laat SW, van Zoelen EJJ. Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. *J Cell Physiol* 1988, **134**, 101–108.
12. Sutherland RL, Hall RE, Pang GYN, Musgrove EA, Clarke CL. Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells. *Cancer Res* 1988, **48**, 5084–5091.
13. Reddel RR, Murphy LC, Sutherland RL. Factors affecting the sensitivity of T-47D human breast cancer cells to tamoxifen. *Cancer Res* 1984, **44**, 2398–2405.
14. Czech MP. Signal transmission by the insulin-like growth factors. *Cell* 1989, **59**, 235–238.
15. Humbel RE. Insulin-like growth factors I and II. *Eur J Biochem* 1990, **190**, 445–462.
16. Steele-Perkins G, Turner J, Edman JC, *et al.* Expression and characterization of a functional insulin-like growth factor I receptor. *J Biol Chem* 1988, **263**, 11486–11492.
17. Hofmann C, Goldfine ID, Whittaker J. The metabolic and mitogenic effects of both insulin-like growth factor are enhanced by transfection of insulin receptors into NIH3T3 fibroblasts. *J Biol Chem* 1989, **264**, 8606–8611.
18. Cullen KJ, Yee D, Sly WS, *et al.* Insulin-like growth factor receptor expression and function in human breast cancer. *Cancer Res* 1990, **50**, 48–53.
19. Lammers R, Gray A, Schlessinger J, Ullrich A. Differential signalling potential of insulin- and IGF-I-receptor cytoplasmic domains. *EMBO J* 1989, **8**, 1369–1375.
20. Derynck R. Transforming growth factor α. *Cell* 1988, **54**, 593–595.
21. Ebner R, Derynck R. Epidermal growth factor and transforming growth factor-α: differential intracellular routing and processing of ligand-receptor complexes. *Cell Regulation* 1991, **2**, 599–612.
22. Muller WJ, Lees FS, Dickson C, Peters G, Pattengale P, Leder P. The *int-2* gene product acts as an epithelial growth factor in transgenic mice. *EMBO J* 1990, **9**, 907–913.
23. Nonomura N, Nakamura N, Uchida N, *et al.* Growth-stimulatory effect of androgen-induced growth factor(s) secreted from Shionogi carcinoma 115 cells on androgen-unresponsive cancer cells in a paracrine mechanism. *Cancer Res* 1988, **48**, 4904–4908.
24. Li S, Shipley GD. Expression of multiple species of basic fibroblast growth factor mRNA and protein in normal and tumor-derived mammary epithelial cells in culture. *Cell Growth and Differentiation* 1991, **2**, 195–202.
25. Musgrove EA, Lee CSL, Sutherland RL. Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor α, epidermal growth factor receptor, c-*fos* and c-*myc* genes. *Mol Cell Biol* 1991, **11**, 5032–5043.
26. Fitzpatrick SL, LaChance MP, Schultz GS. Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. *Cancer Res* 1984, **44**, 3442–3447.
27. Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 1991, **65**, 701–713.
28. Arteaga CL, Coronado E, Osborne CK. Blockade of the epidermal growth factor receptor inhibits transforming growth factor α-induced but not estrogen-induced growth of hormone-dependent human breast cancer. *Mol Endocrinol* 1988, **2**, 1064–1069.

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Clonogenic Potential of Myeloid Leukaemia Cells *In vitro* is Restricted to Leukaemia Cells Expressing the CD34 Antigen

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Cells from patients with acute myeloid leukaemia (AML) or chronic myeloid leukaemia (CML) were separated into CD34-enriched and CD34-depleted subpopulations. The clonogenic capacities of these two subpopulations were then compared to each other and to the original unseparated cell population. In every study, the CD34-enriched subpopulation demonstrated a substantial increase in clonogenicity *in vitro* in comparison with the original cell population, while the reverse was the case for the CD34-depleted subpopulations. For reasons not clear at present, the enrichment for clonogenic cells far exceeded the enrichment for cells expressing the CD34 antigen. Additionally, the clonogenic potential was found to be unrelated to the level of *myc* expression in the various cell populations.
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INTRODUCTION

IN ACUTE myelogenous leukaemia (AML) myeloid maturation is arrested at or near the myeloblast level with essentially all of the leukaemia cells within a patient arrested at the same level of maturation. The leukaemia cells within an individual patient are not, however, biologically equivalent since only a subset will produce clonal growth *in vitro* [1–3] and these have a more "immature" immunophenotype than the non-clonogenic cells [4].

This report demonstrates that as with normal myeloid cells,