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Factors Influencing the Response of MCF-7 Cells to an Agonist of Luteinising Hormone-releasing Hormone

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To clarify the mechanism by which the luteinising hormone-releasing hormone agonist, buserelin, may have direct effects on breast cancer cells, factors potentially influencing its action have been studied in the MCF-7 breast cancer cell line. Oestradiol and epidermal growth factor (EGF), which stimulate the growth of MCF-7 cells in culture, reversed, at least in part, the inhibitory effects of buserelin. Insulin also abolished growth inhibition. Quantitative effects of buserelin differed according to the batch of fetal calf serum used as media supplement. These data suggest that the direct inhibitory effects of buserelin on breast cancer cells are mediated at least in part by an antagonism of growth-promoting factors.
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INTRODUCTION

LUTEINISING HORMONE-RELEASING HORMONE (LHRH) agonist analogues may produce tumour remissions in premenopausal women with advanced breast cancer [1–3]. Suppression of the pituitary–ovarian axis is probably responsible for disease responses in these patients [1, 4, 5] since LHRH agonist therapy reduces circulating oestrogen to levels comparable with those in castrated or postmenopausal women [2, 5]. However, alternative modes of action are possible as LHRH agonists can also benefit postmenopausal patients [2, 6]. Direct antitumour mechanisms may exist and responses to LHRH analogues have been detected *in vitro* using breast tumour cell lines [7–12]. In an attempt to understand more fully the direct actions of LHRH agonist on breast tumour cells, factors which influence response have been investigated.

MATERIALS AND METHODS

Cells

The MCF-7 cells used in this study were originally obtained from the Michigan Cancer Foundation, Michigan. Their characterisation has been described previously by Miller *et al.* [9]. The experiments were conducted using cells from pass numbers 183 to 210. Stock cultures were passaged at 6-day intervals to 50% confluence and maintained under a humidified atmosphere of 5% CO_2:95% air at 37°C in Dulbecco's MEM (50 ml/150 cm^2 culture flask) supplemented with 8 mmol/l glutamine, 4 mmol/l sodium bicarbonate 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Gibco). Media were refreshed every 3 days.

Cell growth experiments

Log phase cultures of MCF-7 cells were collected after culture media removal by washing with 0.02% EDTA in 1 ml phosphate-buffered saline (PBS) followed by incubation with 0.1% trypsin in PBS for 5 min at 37°C. These were subsequently plated out in 60 mm petri dishes at a population density of 0.5×10^6 cells in culture media (4 ml), and supplemented as appropriate with the LHRH agonist buserelin, oestradiol (Steraloids, Croydon, UK), bovine insulin (Sigma) or synthetic mouse epidermal growth factor (EGF) (ICN Biochemicals, High Wycombe, UK) at concentrations described in individual experiments. Cultures were incubated at 37°C for 4 days, all media being renewed daily. Population curves were then constructed from haemocytometer counts obtained at each time point.

Statistical analysis

Results were analysed using two-factor analysis of variance to indicate whether there was an overall significant effect of a particular additive (rather than the effect in an individual experiment). Where such differences were observed, further analysis was carried out using Student's *t* test.

RESULTS

Response to buserelin

As is shown in Fig. 1, the growth of MCF-7 breast cancer cells may be inhibited by buserelin. Agonist at concentrations of 10^{-6} and 10^{-9} mol/l produced a significant decrease in cell number as compared to both control cells and at an agonist concentration of 10^{-11} mol/l ($P < 0.001$). Although exposure to buserelin at concentrations in excess of 10^{-9} mol/l caused cancer cell numbers to decrease, the remaining cells maintained viability and exposure to media free of the peptide allowed cells to display increased growth after an initial lag-phase (Fig. 2).

Conversely, exponential growth phase cells grown in the absence of LHRH agonist for 2 days were susceptible to buserelin, treatment causing a marked reduction in cell number compared with control cells.

Effects of oestrogen, insulin and EGF

Both oestradiol (10^{-10} mol/l) and EGF (10 ng/ml) stimulated the growth of the MCF-7 cell line, causing a significant increase ($P < 0.001$) in cell number over that of the controls (Figs 3a and b, respectively). In contrast, insulin (10 mg/ml) had no apparent effect on the growth rate of the cells (Fig. 3c).

When used in combination with buserelin (10^{-6} mol/l) both

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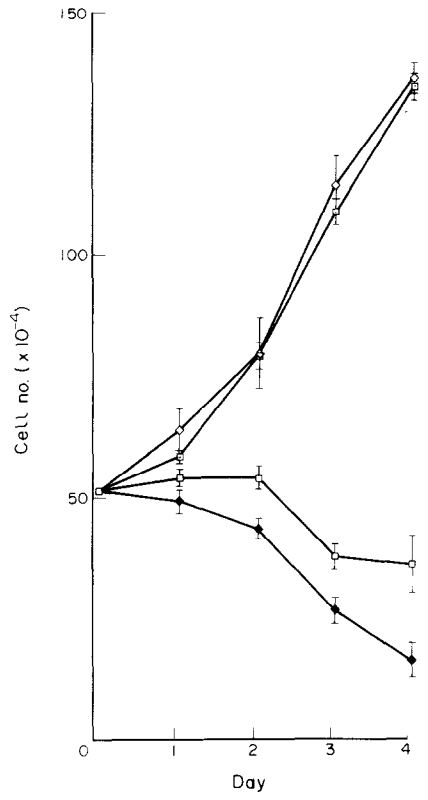

Fig. 1. Suppression of MCF-7 cell growth by buserelin. Cells were harvested from triplicate plates at the time points indicated. Mean (S.D.) of a single representative experiment. Control —⊡—, buserelin 10^{-6} mol/l —◆—, 10^{-9} mol/l —□— and 10^{-11} mol/l —◇—.

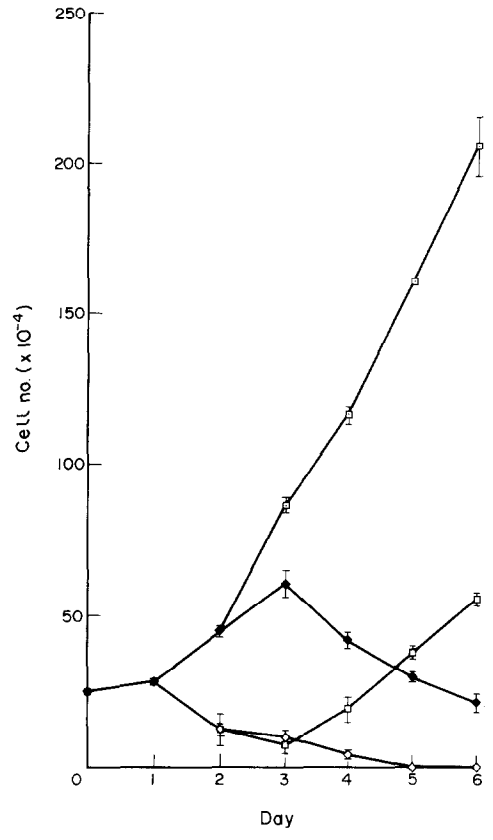

Fig. 2. Growth of MCF-7 cell line in the presence and absence of buserelin. After 48 h, media from triplicate sets of cultures growing in the absence of buserelin (—⊡—) were substituted for media containing the hormone analogue at 10^{-7} mol/l (—◆—). Conversely, one set of triplicate cultures containing buserelin from day 0 (—◇—) were transferred to media free of the agonist at day 2 (□). Mean (S.D.) of a single representative experiment.

insulin and EGF were capable of completely abolishing the growth inhibitory effects of the drug ($P < 0.001$); culture systems containing both buserelin and either EGF or insulin having a similar pattern of growth to cells in the absence of these agents ($P > 0.05$). In contrast, oestrogen only partially reversed the effects of buserelin, cell numbers of the system containing both the LHRH agonist and oestrogen being greater than those containing LHRH agonist alone ($P < 0.05$) but lower than either of those supplemented with oestradiol or those without supplementation.

Influence of serum

To determine the potential effects of fetal calf serum on the response to buserelin, cultures were set up in the presence of 5 different batches of serum. The growth rate of cells in the absence of the drug was similar irrespective of the source of serum. Inclusion of buserelin in the culture media always reduced cell numbers compared with controls. However, the degree of inhibition did vary markedly between batches of serum such that it was possible in the same experiment to demonstrate both profound suppressive effects in which buserelin caused a net reduction in cell numbers over the study period (serum A, Fig. 4) and less marked responses in which cell number still increased in the presence of buserelin (serum B, Fig. 4).

DISCUSSION

The results of this study confirm and extend our published observation that buserelin can suppress the growth of certain clones of MCF-7 breast cancer cells in monolayer culture [9]. Evidence is presented that oestrogen, EGF, insulin and serum are important factors in determining the cellular response to this agonist.

Oestradiol at a concentration of 10^{-10} mol/l not only stimulated the growth of MCF-7 cells but partially overcame the inhibitory effects of the LHRH agonist. It is important to note that although oestrogen incompletely reversed the effect of buserelin, the net effect of adding the LHRH agonist to oestrogen-stimulated cells is still one of inhibition. This would be consistent with the observation of Foekens *et al.* [13] who noted that buserelin markedly inhibited the growth of oestrogen-stimulated MCF-7 cells. The mechanism by which a small polypeptide such as an LHRH agonist can interact antagonistically with oestrogen is unknown but it may be pertinent that the mitogenic effects of oestrogen on MCF-7 cells grown in culture may be mediated at least in part by induction of growth factors such as transforming growth factor α (TGF-α) and the insulin-like growth factors (IGF) [14,15]. It is thus relevant that both EGF and insulin were also able to abolish the inhibitory effects of buserelin, since both EGF and TGF-α bind to the EGF receptor and insulin and IGF may interact with IGF receptors which can be shown to be present on MCF-7 cells [15]. It is therefore tempting to speculate that the antiproliferative effects of buserelin on MCF-7 cells are mediated through a blockage of autocrine growth factors such as TGF-α and IGF-I and IGF-II.

Recent studies on the CG5 breast cancer cell line have yielded similar results [12] in that the antiproliferative effects of LHRH agonists were against oestrogen-stimulated cells or those exposed to other mitogens, such as EGF or insulin.

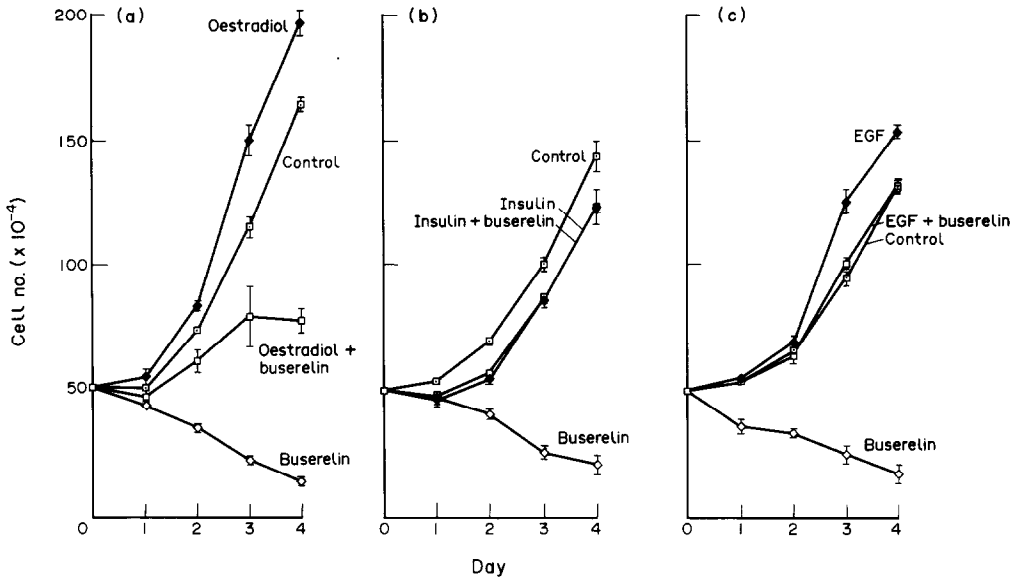

Fig. 3. The influence of (a) oestradiol, (b) insulin and (c) EGF on MCF-7 breast tumour cells both in the presence and absence of LHRH agonist. Mean (S.D.) of a single representative experiment. Control —⊡—, buserelin 10^{-6} mol/l —◇—, modifying factor [(a) oestradiol 10^{-10} mol/l, (b) insulin 10 mg/ml, (c) EGF 10 ng/ml] —◆—, modifying factor [(a) oestradiol 10^{-10} mol/l, (b) insulin 10 mg/ml, (c) EGF 10 ng/ml] + buserelin 10^{-6} mol/l —□—.

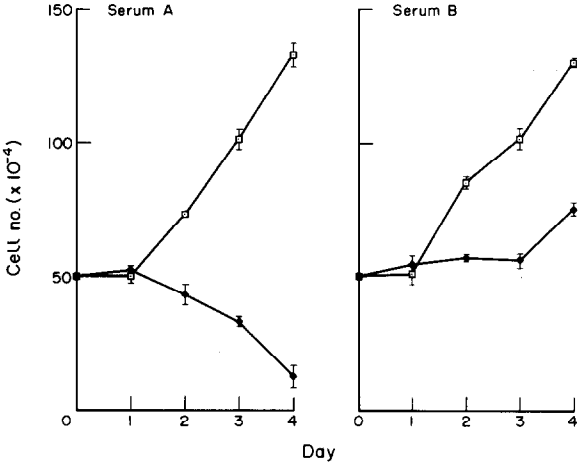

Fig. 4. The inhibitory effect of LHRH agonist on MCF-7 cells grown in media containing different sera. Cells were grown in the absence (—⊡—) or presence (—◆—) of LHRH agonist (10^{-6} mol/l). Mean (S.D.) of a single representative experiment.

It seems likely that the variation in response to buserelin seen by culturing with different batches of serum is caused by the presence of different concentrations of mitogenic factors within the serum. Whilst we do not have definite proof of this, fetal calf serum from different sources is likely to contain widely different concentrations of growth factors which might survive heat denaturation. The fact that significant differences were not observed in the growth rate of control cells when cultured in different batches of serum does not exclude this possibility. Thus, in this study, insulin completely abolished the effects of buserelin but did not influence the growth rate of MCF-7 cells. However, the observation that insulin and serum can reduce the sensitivity of cells to buserelin would, in part, explain the difficulties of others to demonstrate inhibitory effects of these drugs [16].

Whilst the inhibitory effects of LHRH agonists in MCF-7 cells have been interpreted as anti-oestrogenic [13], similar marked inhibitory effects have been observed against prolactin-stimulated growth of T-47D cells [7]. This suggests that the actions of LHRH agonists are directed against stimulated cells, the nature of the stimulation being specific for different cell lines, i.e. oestrogen in MCF-7 cells and prolactin for T-47D cells. Our finding that buserelin inhibition of MCF-7 cells is associated with accumulation of cells in G_0/G_1 [17] would be compatible with LHRH agonists having an antagonistic action against factors which normally commit to the cell cycle. If so, then it is possible that LHRH analogues may have a therapeutic role beyond that of simply causing medical castration in patients. With a greater understanding of the mechanism by which LHRH agonists interact with growth factors and modulators, this wider potential may yet be exploited to the benefit of cancer patients.

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Micrometastatic Tumour Cells in Bone Marrow of Patients with Gastric Cancer: Methodological Aspects of Detection and Prognostic Significance

Günter Schlimok, Ilona Funke, Klaus Pantel, Friedrich Strobel, Fritz Lindemann, Jens Witte and Gert Riethmüller

Monoclonal antibodies (Mab) are potent probes to identify individual tumour cells or small tumour cell clusters in bone marrow. In the present study, various antibodies directed against either cell surface or intracytoplasmic antigens of epithelial cells were assessed for their ability to detect such cells in bone marrow of patients with breast, colorectal and gastric cancer. According to the presented data, monoclonal antibodies against intracellular cytokeratin (CK) components are superior in terms of specificity and sensitivity to antibodies reacting with epitopes of the cell membrane. Using a monoclonal antibody against the cytokeratin polypeptide 18 in connection with the alkaline phosphatase anti-alkaline phosphatase detection system (APAAP), we could detect tumour cells in bone marrow of 34 out of 97 patients with gastric cancer examined at the time of primary surgery. The incidence of positive findings was correlated to established risk factors, such as histological classification and locoregional lymph node involvement. Clincal follow-up studies on 38 patients demonstrated a significantly increased relapse rate in patients presenting with CK-positive cells in their bone marrow at the time of primary surgery. Thus the described technique may help to identify patients with gastric cancer carrying a high risk of early relapse.
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INTRODUCTION

THE TREATMENT of the most common types of malignant tumours has hardly improved during the last decade. This overall negative balance is mainly due to the systemic dissemination of tumour cells occurring often prior to the diagnosis or resection of the primary tumour. Thus, the detection and elimination of micrometastases in patients with small resectable tumours poses a major challenge to cancer research.

Monoclonal antibodies (Mab), directed against epithelial differentiation antigens are able to identify individual epithelial tumour cells or small carcinoma cell clusters in mesenchymal organs, which are undetectable by conventional diagnostic methods. The bone marrow presents as an easily accessible mesenchymal organ and tissue compartment from which epithelial cells appear to be rigorously excluded in non-malignant conditions. It is evident that an immunocytochemical assay depends on the specificity of the antibody and on the sensitivity of the applied detection system. The antibody used should be