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Interaction between Hormone-dependent and Hormone-independent Human Breast Cancer Cells

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We developed two different models based on *in vitro* co-culture of hormone-dependent and hormone-independent cell lines to simulate the cell population heterogeneity of human breast cancer tumours. Oestrogen-dependent (MCF-7, ZR 75.1) and oestrogen-independent cell lines (MDAMB-231 BT-20) were grown under serum-free conditions. Co-culture of hormone-dependent and hormone-independent cell lines resulted in an increased cell yield compared to single cell cultures carried out at the same seeding ratios. Such an increase was not affected by addition of oestradiol and single growth factors (EGF, bFGF and IGF-I). These results allow us to conclude that in a heterogeneous cell population like human breast tumours, interaction between hormone-dependent and hormone-independent cell lines may result in a complex regulation of cell growth.
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INTRODUCTION

DURING THE last decade many cell lines have been characterised and used to investigate the growth modality of breast cancer [1]. On the basis of receptor status and of their dependence on oestrogens, cell lines have been subdivided into endocrine-dependent and endocrine-independent and have been separately investigated. However, human breast tumours are a heterogeneous mixture of different cell populations, which are characterised by variable degrees of hormone dependence [2], so that the growth of a tumour may be the result of the individual properties of cell subpopulations and of their interaction. Endocrine-dependent cells are oestrogen-receptor positive, and their proliferation rate is increased by the addition of oestradiol [3, 4]. They do not constitutively produce growth factors (GFs), but after administration of oestradiol (E_2) an induction of GFs has been observed [5, 6]. Conversely, endocrine-independent cell lines produce GFs constitutively and do not increase their proliferation rate as a response to E_2 [7]. This would imply that in the absence of hormones, oestrogen receptor positive (ER+) cells can be induced to proliferate by GFs released by oestrogen receptor negative (ER−) cells present in the heterogeneous tumour cell population. Indeed, it is well known that GFs such as epidermal (EGF) basic fibroblast (bFGF) and insulin-like GF (IGF-I) can stimulate ER+ cells to proliferate also in the absence of E_2 [8].

To improve knowledge on this biological aspect, we studied the interaction between ER− and ER+ breast cancer cell lines under several culture conditions. The study was performed by using mixed cell cultures to allow cell contact or transwell systems to limit cell interaction to diffusible substances.

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MATERIALS AND METHODS

Chemicals

DMEM/F12 1:1 without phenol red, transferrin, sodium selenite, bovine serum albumin V, 3,3',5-triiodotyronine, glutathione, ethanolamine, 17β-oestradiol and collagen type IV were from Sigma. Penicillin, streptomycin and amphothericin B were from Whitaker M.A. Bioproducts (Walkersville, Maryland). EGF and bFGF were obtained from Collaborative Research (Two Oak Park, Bedford, Massachusetts) and IGF-I from Imcera Mallinckrodt (St Louis, Missouri). All other chemicals were of analytical grade.

Cell lines

Human breast cancer cell lines were kindly provided by K. Horwitz, University of Colorado (MCF-7), by G. Daxenbichler, University of Innsbruck (BT-20), by M.J. Siciliano, M.D. Anderson Hospital and Tumor Institute (MDAMB-231) and by F. Fox, UCLA, Los Angeles (ZR 75.1).

Media

Cells were maintained in 1:1 DMEM/F12 without phenol red in the presence of 5% fetal calf serum. Experiments were performed in phenol-red-free DMEM/F12 containing 10 μg/ml transferrin, 10 ng/ml sodium selinite, 20 μg/ml glutathione, 0.3 nmol/l 3,3',5-triiodotyronine, 50 μmol/l ethanolamine and 200 μg/ml bovine serum albumin (MOM-3).

Mixed cell experiments

ER+ and ER− cells were mixed under specific proportions, plated in 25 cm^2 flasks at a density of 800000 cells/flask or at a density of 300000 in co-culture experiments with MCF-7 cells and cultured for 7 days in different media. Single cell lines were run in parallel at the same inoculation density and culture conditions. Cells were harvested with trypsin/EDTA and collected by detaching with a scraper. Experiments were stopped when cells were exponentially growing. Cell number was determined by counting a small aliquot in a Burker chamber; the bulk of cells was fixed in 70% ethanol for DNA flow cytometry.

Transwell co-culture experiments

ER+ cell lines were plated in type IV collagen-treated (4 $\mu g/cm^2$) 6-well cluster dishes. Plating density was 100000 and 250000 cells/well, respectively, for MCF-7 and ZR 75.1 cells. ER− cells (MDAMB-231 and BT-20) were grown on collagen-coated transwell membranes (Costar Corporation, Cambridge, Massachusetts) at a density of 500000 cells/transwell. After 24 h, the transwells were placed on the top of the well using sterile tweezers. Culture media were changed at day 4. At day 7, media were removed and DNA content was determined directly in the wells using diphenilamine (Merck) essentially according to Burton [9].

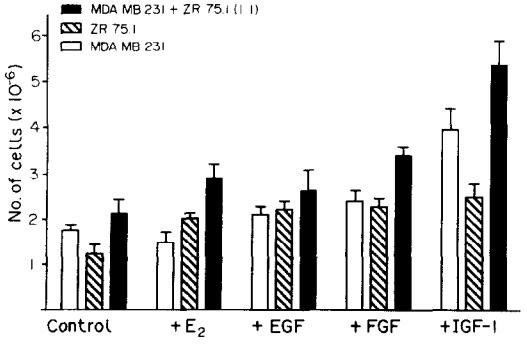

Fig. 1. Total cell number after 7 days of culture of MDAMB-231, ZR 75.1 and MDAMB-231 mixed with ZR 75.1. Single cell line cultures and co-culture results are represented for five different experimental conditions, i.e. in the basal medium MOM-3, and in the presence of 10^{-8} mol/l E_2, 1 ng/ml bFGF and 10 ng/ml IGF-I. Results are the mean of triplicate determinations.

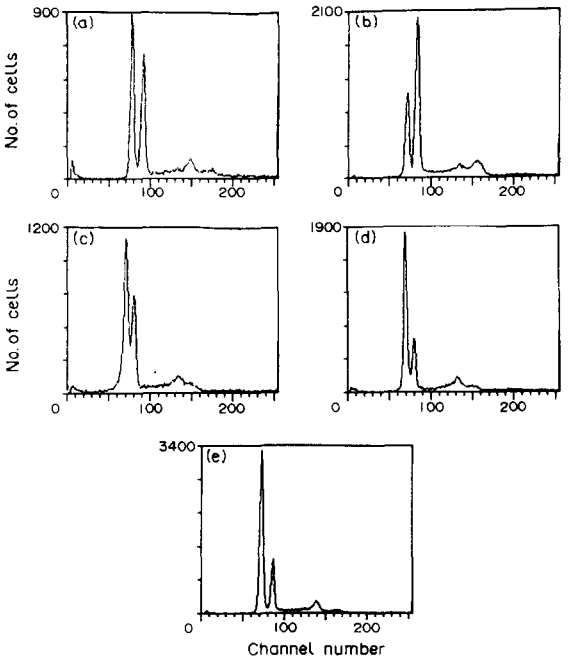

Fig. 2. DNA histograms of cell samples obtained after co-culture of MDAMB-231 in the basal medium (a), in the presence of 10^{-8} mol/l E_2 (b) and in the presence of 10 ng/ml EGF (c), 1 ng/ml bFGF (d) and 10 ng/ml IGF-I (e). Histograms are representative of three separate experiments. The coefficients of variation of the G_0/G_1 peaks were below 5% in all experiments.

Flow analysis of cellular DNA content

Samples of 10^6 ethanol-fixed cells were stained with propidium iodide (50 μg/ml, Sigma) RNase (100 μ/ml, Sigma) and Nonidet P40 (0.05%) overnight at 4°C [10]. The samples were briefly sonicated to disaggregate the clumps and passed through a 70 μm filter. A number of cells ranging from 20000 to 50000 was analysed for each sample by FACScan (Becton Dickinson).

RESULTS

Flow cytometric analysis showed a higher G_0/G_1 DNA content in ER+ than in ER− cell lines (data not shown). Figure 1 shows results obtained by co-culture of ZR 75.1 and MDAMB-231. The total cell number observed for the single cell cultures was a function of the medium, as already observed in a previous study [11]. However, under all culture conditions, mixed cell cultures were characterised by a higher final cell number than the single cell lines. Qualitative flow ($P<0.01$) cytometric analysis (Fig. 2) showed two significantly distinct G_0/G_1 peaks in co-cultures in basal medium conditions. This finding guarantees the possibility of resolving the peaks belonging to the two subpopulations and also shows that in the case of the ZR 75.1 and MDAMB-231 cell co-culture the growth advantage observed as compared to the single cell line cultures is the result of contribution by both cell lines.

When the two cell lines were co-cultured in the presence of

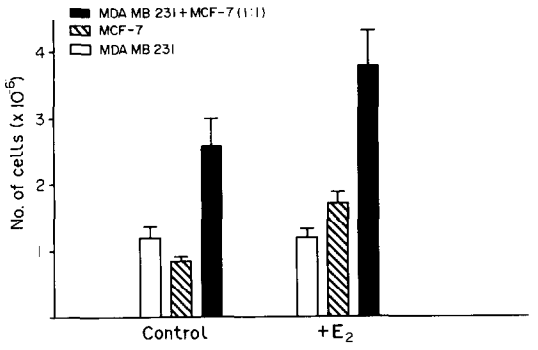

Fig. 3. Total cell number after 7 days of culture of MDAMB-231, MCF-7 and MDAMB-231 mixed with MCF-7. Experiments were run in two different conditions, i.e. in the basal medium MOM-3 and in the presence of 10^{-8} mol/l E_2. Results are the mean of triplicate experiments.

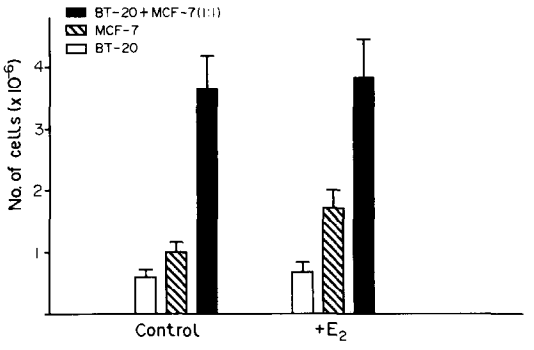

Fig. 5. Total cell number after 7 days of culture of BT-20, MCF-7 and of BT-20 mixed with MCF-7 with or without 10^{-8} mol/l E_2. Results are the mean of triplicate determinations.

E_2, a cell number increase could still be demonstrated (Fig. 1) and DNA flow cytometric analysis indicated a prevelance of ER+ cells over ER− cells. In contrast, when different GFs were added to the basal medium (EGF, 10 ng/ml; bFGF 1 ng/ml; IGF-I, 10 ng/ml) the flow cytometric analysis revealed a prevalence of MDAMB-231 cells (Fig. 2). Co-culture of MDAMB-231 and MCF-7 cells (Fig. 3) resulted into a dramatic increase of cell number ($P<0.001$ determined by Student's t test) and, as can be seen from DNA flow cytometry results (summarised in Table 1) the peak corresponding to the MDAMB-231 cells is very small due to the massive growth of MCF-7 cells; this latter is also confirmed in the transwells experiments (see below). Figures 4 and 5 show the results obtained by mixing BT-20 cells and ZR 75.1 and MCF-7 cells, respectively. Again, co-culture resulted in a significantly increased growth as compared to single cell lines. This finding was consistently observed in basal medium and in the presence of E_2. Moreover, analysis of the correspondent DNA content profiles (Table 1) showed that the growth advantage for mixed MCF-7 and BT-20 was maximally ascribable to growth of the ER+ cell line. The growth pattern was not affected by the presence of E_2. Conversely, when ZR 75.1 cells were mixed with BT-20 cells, the ER− cell line was prevalent in the basal medium whereas a stimulation of the ER+ cell line was observed in the presence of E_2 (Table 1).

The two ER+ cell lines MCF-7 and ZR 75.1 were cultured using the transwell system with the same ER+ cell line or with the two ER− cell lines MDAMB-231 and BT-20. Control experiments were run in the absence of transwells. ZR 75.1 was not affected by the presence of the same cell line or by BT-20 cells, whereas a significant increase in cell number was induced by MDAMB-231 ($P<0.01$). Similarly, MCF-7 cells were not affected by the presence of the same cell line, but a significant stimulation was observed in the presence of MDAMB-231 ($P<0.01$) and BT-20 ($P<0.01$) (Fig. 6).

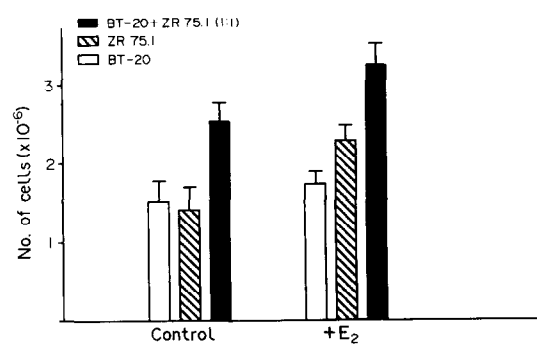

Fig. 4. Total cell number after 7 days of culture of BT-20, ZR 75.1 and of BT-20 mixed with ZR 75.1 with or without 10^{-8} mol/l E_2. Results are the mean of triplicate determinations.

DISCUSSION

In this study, consequences of interaction between ER− and ER+ cell lines on total cell growth were studied using mixed cell cultures to allow cell contact and transwell systems to prevent direct cell contact and allow free diffusion of cell-secreted factors. It was observed that all mixed ER+ and ER− cell line cultures were characterised by a growth advantage compared to the corresponding single cell lines, regardless of the addition of E_2 to the medium. Addition of GFs increased the proliferation rate of MDAMB-231 and ZR 75.1 separately grown as well as of mixed cell cultures, but with a higher advantage for the latter. This does not rule out the secretion of GFs as a possible mechanism for increased cell growth in the mixed cell cultures, but would suggest that other factors resulting from cell-to-cell interactions should be considered as well. Since we tested only three GFs and at a single concentration, it cannot be excluded that different concentrations of these peptides or other GFs are able to abolish the additional increase in cell proliferation observed in co-cultures. Interestingly, the growth advantage observed for co-cultures in all the various cell line combinations was confirmed by the corresponding transwell co-culture experiments in all cases except for the

Table 1. Relative proportions between ER positive and ER negative cells lines after co-culture

	Co-culture in	
Cell lines	MOM3	MOM3 + 10^{-8} mol/l E_2
MDAMB-231/MCF-7	7/40	ND/46
MDAMB-231/ZR75.1	35/26	28/32
BT-20/MCF-7	5/45	3/61
BT-20/ZR75.1	24/10	24/16

*Relative proportions have been calculated from the G_0/G_1 peaks of the corresponding DNA histograms. The coefficient of variation was less than 5% in all cases.
ND = not detectable.

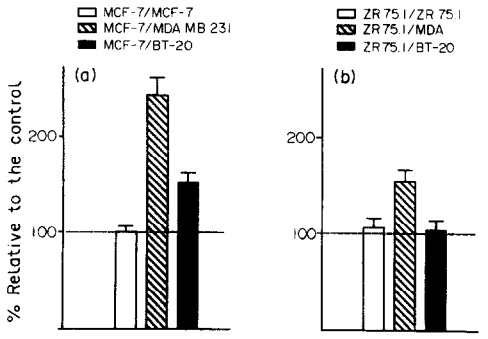

Fig. 6. Co-culture experiments with the transwell system. (a) MCF-7 total cellular DNA content expressed as percentage variation with respect to the controls (cells grown in the absence of transwells) when the cells were grown in the presence of transwells containing, respectively, MCF-7 MDAMB-231 and BT-20 cells. (b) ZR 75.1 total cellular DNA content as percentage variation with respect to the controls when the cells were grown in the presence of transwells containing, respectively, ZR 75.1, MDAMB-231 and BT-20 cells. The results are representative of three separate experiments.

ZR 75.1 and BT-20 combination. Such results would indicate that besides diffusible factors, also close cell-to-cell contact is important for proliferative stimulation. Alternatively, growth stimulation may not be necessarily caused by the ER− cells, and the opposite situation might also be possible. These two hypotheses are presently under study.

The complex interaction between heterogenous cell subpopulations could explain the variability in tumour progression among the different patients and the failure in response to endocrine treatment for patients with ER+ tumours. Cell interactions have been postulated by Dickson *et al.* [12] following the evidence of GF production by E_2 and that hormone-dependent cells may be stimulated to growth in nude mice by concentrates of conditioned media derived from breast cancer cells. However, in a successive study, no paracrine effects were observed by Osborne *et al.* [13] following injection of a mixture of ER+ and ER− cell into nude mice and through DNA flow cytometric analysis. Robinson and Jordan [14], using the transwell system, were able to demonstrate a stimulation of MCF-7 cells by the MDAMB-231 similar to that observed in our experiments. They also reported that MCF-7 cells failed to respond to anti-oestrogens when co-cultured with MDAMB-231, probably due to the secretion of GFs. In fact, Cormier and Gordon [15] had already demonstrated that tamoxifen was no longer able to inhibit the growth of anti-oestrogen-responsive cells if they were stimulated by EGF.

The results obtained in our study are therefore in agreement with those reported by Lippman *et al.* [5] and Robinson and Jordan [14] by using different experimental approaches. Moreover, from our data it appears that interaction between endocrine-dependent and endocrine-independent cells can vary in relation to the specific cell lines used. It is finally noteworthy that the cross stimulation observed between mixed cultures of hormone-dependent and hormone-independent cell lines is not necessarily due to the ER− cells, as hypothetically assumed in previous studies. The contribution of stromal cells to the paracrine stimulation of epithelial cells should not be underestimated and it will be the object of future research.

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