Overexpression of insulin-like growth factor II (IGFII) in ZR-75-1 human breast cancer cells: higher threshold levels of receptor (IGFIR) are required for a proliferative response than for effects on specific gene expression

K. Abdul-Wahab, D. Corcoran, A. Perachiotti and P.D. Darbre

Division of Cell and Molecular Biology, School of Animal and Microbial Sciences, The University of Reading, Reading, UK

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Abstract. Previous transfection experiments using a zinc-inducible expression vector have shown that overexpression of insulin-like growth factor II (IGFII) in MCF7 human breast cancer cells can reduce dependence on oestrogen for cell growth *in vitro* (DALY RJ, HARRIS WH, WANG DY, DARBRE PD. (1991) *Cell Growth Differentiation* 2, 457–464.). Parallel transfections now performed into another oestrogen-dependent human breast cancer cell line (ZR-75-1) yielded three clones of transfected ZR-75-1 cells that produced levels of zinc-inducible *IGFII* mRNA and secreted mature IGFII protein similar to those found in the transfected MCF7 cells. However, unlike in MCF7 cells, no resulting effects were found on cell growth in the ZR-75-1 clones, even though the ZR-75-1 clones possessed receptors capable of binding ^{125}I -IGFI and showed a growth response to exogenously added IGFII. Medium conditioned by the ZR-75-1 clones could stimulate growth of untransfected MCF7 cells, indicating that the secreted IGFII protein was bioactive. Furthermore, zinc-induced IGFII was capable of increasing both *pS2* mRNA levels and CAT activity from a transiently transfected *API-CAT* gene in the ZR-75-1 clones. Constitutive co-overexpression of the protein processing enzyme PC2 resulted in reduced levels of large forms of zinc-inducible IGFII, but zinc treatment still produced no effect on cell growth rate. Finally, however, constitutive co-overexpression of the type I IGF receptor (IGFIR) did result in zinc-inducible increased basal cell growth and reduced dependence on oestrogen for cell growth. These results demonstrate that while overexpression of IGFII *per se* was sufficient to deregulate MCF7 cell growth, the ZR-75-1 cells are limited in their proliferative response by their intrinsic receptor levels. However, although the proliferative response was limited, molecular responses (expression of *pS2* and *API-CAT*) were not limited, indicating that different cellular responses can have different threshold receptor level requirements.

Correspondence: P. D. Darbre, Division of Cell and Molecular Biology, School of Animal and Microbial Sciences, The University of Reading, Whiteknights, PO Box 228, Reading RG6 6AJ, UK. E-mail: p.d.darbre@reading.ac.uk

INTRODUCTION

Studies in cell culture have shown that the growth of breast cancer cells is regulated by an intricate array of steroid hormone, peptide hormone and growth factor pathways (Lippman & Dickson 1990, Gee, McClelland & Nicholson 1996) which appear to exhibit considerable cross-talk in their actions (Kenney & Dickson 1996). Of the many components, interactions between oestrogen (Miller 1996) and insulin-like growth factors (IGFs) (Yee 1998) appear to be particularly important. Oestrogen is a major stimulus for the growth of breast cancer cells and current endocrine therapy of breast cancer depends on the maintenance of oestrogen-responsive growth (Miller 1996). Progression of breast cancer cells to an oestrogen-unresponsive state is a major clinical problem for which detailed molecular mechanisms remain unclear. In some cases, there may be loss of (Miller 1996) or alterations to (Horwitz 1994, Castles & Fuqua 1996) the oestrogen receptor, but in other cases, there can be loss of the growth response to oestrogen without any loss of functional oestrogen receptors (Darbre & Daly 1990). The steroid deprivation cell culture model shows that long-term growth without steroid results in cell adaptation through an increase in non-oestrogen-stimulated cell growth rate, which can be associated with alterations to growth factor production or sensitivity (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Daly & Darbre 1990, Daly, King & Darbre 1990, Darbre & Daly 1990), but the specific role of IGFs in these processes remains unclear.

Studies of overexpression of components of the IGF system in cell culture models have demonstrated mitogenic effects in MCF7 cells following overexpression of ligand (IGFII) (Daly *et al.* 1991; Cullen *et al.* 1992) but surprisingly moderate effects with overexpression of receptor (IGFIR) (Daws, Westley & May 1996, Guvakova & Surmacz 1997). Overexpression of components of other growth factor signalling pathways have produced varied effects on oestrogen sensitivity of breast cancer cell growth. In terms of ligand overexpression, overexpression of heregulin- β 2 (Tang *et al.* 1996), fibroblast growth factor 4 (Mcleskey *et al.* 1993) and transforming growth factor β 1 (Arteaga *et al.* 1993) could all produce some form of oestrogen-independent growth *in vitro* and/or *in vivo*. However, the ability of a single growth factor to abrogate the need for oestrogen in breast cancer cell growth was not always evident since overexpression of TGF α did not alter oestrogen regulation of MCF7 cell growth (Clarke *et al.* 1989). This is surprising in view of the results with heregulin and since epidermal growth factor (EGF)-like ligands do play a role in growth of breast cancer cells (Lippman & Dickson 1990, Gee *et al.* 1996, Kenney & Dickson 1996, Miller 1996). Furthermore, experiments in a different steroid sensitive system showed that TGF α overexpression could override glucocorticoid suppression of Con8 mammary cells (Goya *et al.* 1993). In terms of overexpression of receptors, overexpression of epidermal growth factor receptor (EGFR) in ZR-75-1 cells showed no effect on basal growth of cells in serum-containing medium (Van Agthoven *et al.* 1992). This is again disappointing in view of the fact that members of the EGFR family are well known to be overexpressed in oestrogen receptor (ER) negative breast tumours (Foekens *et al.* 1996) and this overexpression is currently being exploited as a novel therapeutic target (Groner, Wels & Hynes 1996). The *erbB2* oncogene encodes a growth factor receptor which has extensive homology with the EGFR and overexpression of *c-erbB2* was found to confer resistance to the anti-oestrogen, tamoxifen, in hormone sensitive breast cancer cells (Benz *et al.* 1992) and in the EGFR overexpressing MCF7 cells, there was an interesting tendency for progression to an ER-independent phenotype when grown with anti-oestrogen (Van Agthoven *et al.* 1992).

These studies raise interesting questions about the relative importance of different ligands and of ligand vs. receptor in the growth of breast cancer cells. However, since work to date has centred on only one cell line for each transfection experiment, it is not possible to ascertain whether effects represent a general rule or reflect the specific context of that one cell line. The work described here has aimed to extend observations on overexpression of IGFII in MCF7 cells (Daly *et al.* 1991) into another oestrogen-dependent breast cancer cell line ZR-75-1. Clones of IGFII transfected ZR-75-1 cells were shown to produce levels of IGFII mRNA and secreted IGFII protein similar to those found in the MCF7 cells, but unlike the situation in MCF7 cells, no resulting effects were found on cell growth until IGFIR was simultaneously overexpressed. This emphasizes the importance of cell context in overexpression studies, and demonstrates the danger in drawing general conclusions, from work based on one cell line, about the overall importance of one single growth factor or one single growth factor receptor to breast cancer cell growth.

MATERIALS AND METHODS

Expression vectors

The expression plasmid pΔMT12 consisted of the coding sequence for *prepro-IGFII* under control of the human metallothionein IIA promoter with termination signals from SV40 as described previously (Daly *et al.* 1991). The expression plasmid pSRα/PC2 consisted of full-length cDNA for the human PC2 gene (Smeekeens & Steiner 1990) under control of the SR promoter with SV40 termination signals (from P. J. Lowry). The expression plasmid pCMV5 consisted of full-length cDNA for the human IGFIR gene (Steele-Perkins *et al.* 1988) subcloned into pTZ18R vector (Pharmacia, Sweden) under control of the human cytomegalovirus promoter and with SV40 termination signals (from R.E. Lewis). The pSV2neo selection vector has been described elsewhere (Southern & Berg 1982) and the pREP4 vector for hygromycin selection was obtained from a commercial source (Invitrogen, San Diego, CA, USA).

Cell lines

The ZR-75-1 human breast cancer cells were kindly provided by Professor M. Lippman (Engel *et al.* 1978). MCF7 McGrath human breast cancer cells were kindly provided by Dr K. Osborne at passage number 390 (Osborne, Hobbs & Trent 1987). All MCF7 cells used in these studies were of the MCF7 McGrath subline but will be referred to as MCF7 in the text for brevity. MI7 cells are a clone of MCF-7 human breast cancer cells stably transfected with the expression vector pΔMT12 (Daly *et al.* 1991). Administration of 5×10^{-5} M zinc to MI7 cells increases IGFII gene expression (Daly *et al.* 1991), IGFII protein secretion (Lee, Darbre & King 1994) and cell growth rate (Daly *et al.* 1991). Since this increased cell growth can be blocked by the α IR3 antibody to the IGFIR, effects of upregulated IGFII are presumed to act through an autocrine pathway (Daly *et al.* 1991).

Stock culture of human breast cancer cells

Stock ZR-75-1 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Paisley, UK) supplemented with 5% fetal calf serum (FCS) (Gibco BRL) and 10^{-8} M oestradiol (Steraloids, Croydon, UK) in a humidified atmosphere of 10% carbon dioxide in air at 37°C. Oestradiol was dissolved in ethanol and diluted 1/10 000 in culture medium. MCF7 cells were grown routinely as monolayer cultures in DMEM supplemented with 5% FCS, 10^{-8} M oestradiol and 10 µg/ml insulin (Sigma, Poole, UK).

MI7 cells were grown routinely as monolayer cultures in DMEM supplemented with 5% FCS, 10^{-8} M oestradiol and 100 μ g/ml G418 sulphate (Gibco BRL). All cell stocks were subcultured at weekly intervals by suspension with 0.06% trypsin–0.02% EDTA (pH 7.3).

Transfection procedure

ZR-75-1 cells were plated at a density of 3.0×10^5 cells per 9-cm tissue culture dish in stock medium as above. Six days later, cells were transfected for 6 h with 20 μ g of p Δ MT12 and 2 μ g of pSV2neo per dish using the calcium phosphate co-precipitation method (Wigler *et al.* 1979). The cells were washed with DMEM, treated with 25% glycerol in DMEM for 1 min, washed again and incubated overnight in stock medium. Two days later, the medium was changed to stock medium containing 200 μ g/ml G418 sulphate and 3 days later the concentration of G418 sulphate was increased to 1 mg/ml. Selection was continued at this concentration of G418 sulphate until individual clones could be isolated using cloning rings. Transfected clones were routinely maintained as described for MI7 cells above.

ZRI16 cells were plated at a density of 14×10^5 cells per 15-cm tissue culture dish in stock medium as for MI7 cells above. Six days later, cells were co-transfected for 6 h with 100 μ g of either pSR α /PC2 or pCMV5 expression plasmid and 10 μ g of pREP4 selection vector per dish using the calcium phosphate co-precipitation method (Wigler *et al.* 1979). The cells were washed with DMEM, treated with 25% glycerol in DMEM for 1 min, washed again and incubated in stock MI7 medium. After 72 h, the medium was changed to DMEM with 5% FCS, 10^{-8} M oestradiol and 300 μ g/ml hygromycin-B (Sigma). Approximately 3 weeks later, hygromycin-B resistant clones could be isolated using cloning rings. Transfected clones were routinely maintained in DMEM containing 5% FCS, 10^{-8} M oestradiol, 100 μ g/ml G418 sulphate and 60 μ g/ml hygromycin-B.

Cell growth experiments

Cells were suspended from stock plates by treatment with phenol red-free 0.06% trypsin–0.02% EDTA (pH 7.3), added to an equal volume of phenol red-free RPMI 1640 medium (Gibco BRL) containing 5% dextran–charcoal stripped FCS (DCFCS) (Darbre *et al.* 1983) and counted on a haemocytometer. Cells were then added to the required volume of phenol red-free RPMI 1640 medium containing 5% DCFCS at a concentration of 0.2×10^5 cells/ml and plated in monolayer in 0.5-ml aliquots into 24 well plastic tissue culture dishes (Nunc, Roskilde, Denmark). After 24 h, the medium was changed to phenol red-free RPMI 1640 medium supplemented with the appropriate concentration of DCFCS, oestradiol, IGFII (Bachem, Saffron Waldon, UK), and zinc chloride. Culture medium was changed routinely every 3–4 days in all experiments. Cell counts were performed by counting released nuclei on a model ZBI Coulter Counter, as described previously (Daly & Darbre 1990).

RNA extraction and Northern blotting

Cells for RNA extraction were suspended in phenol red-free RPMI 1640 medium containing 5% DCFCS at a concentration of 0.2×10^5 cells/ml and plated in 16 ml aliquots into 9-cm plastic tissue culture dishes (Nunc). After 24 h, the medium was changed to phenol red-free RPMI 1640 medium supplemented with the appropriate concentration of DCFCS, oestradiol, IGFII and zinc chloride. After 5 days in culture, cells were washed *in situ* with isotonic saline, harvested into ice-cold isotonic saline using a rubber policeman and pelleted by centrifugation. Whole cell RNA was prepared by the guanidinium–caesium chloride method (Sambrook, Fritsch & Maniatis 1989) and analysed by Northern blotting using methods and DNA probes exactly as described previously (Daly & Darbre 1990).

Western immunoblotting of IGFII

Cells were plated onto 3.5-cm plastic tissue culture dishes in phenol red-free RPMI 1640 medium supplemented with 5% DCFCS with or without 5×10^{-5} M zinc chloride as required, at a density of 1×10^5 cells/ml. After 24 h, the medium was replenished. After a further 4 days, medium was removed, cells washed twice with phenol red-free RPMI 1640 medium and 1 ml serum-free medium added to each dish. Serum-free medium consisted of phenol red-free RPMI 1640 medium with 15 mM HEPES buffer (Gibco BRL), 5 μ g/ml transferrin (Sigma), 0.75 μ g/ml fibronectin (Sigma) with or without 5×10^{-5} M zinc chloride. After a further 48 h, conditioned medium was collected from each dish, centrifuged briefly to remove cell debris and stored at -70°C . Cells on each dish were counted on a Coulter counter as described above. Proteins were separated by 15% polyacrylamide SDS gel electrophoresis. To each gel track was loaded the volume of conditioned medium equivalent to the same number of cells diluted $\times 2$ with non-reducing sample buffer (26 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.01% w/v bromophenol blue). Proteins were transferred onto PVDF membranes (Immobilon-P, Millipore, Watford, UK) using a wet transfer blotting system (BioRad, Hercules, CA, USA) in 0.05 mM Tris-HCl pH 9.2/0.04 mM glycine/15% (v/v) methanol at 20 V for 18 h at 4°C . The membrane was blocked with phosphate-buffered saline (PBS) (170 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4)/0.05% Tween 20 (PBST) for 1 h, and immunoblotted with a 1 : 25 000 dilution of monoclonal mouse anti-rat IGFII antibody (LabTech, Easons Green, UK) in PBST for 1 h 30 min at room temperature. After washing 6×5 min with PBST, the membrane was incubated with a 1 : 2500 dilution of biotinylated rabbit anti-mouse antibodies (Dako, Copenhagen, Denmark) in PBST for 1 h at room temperature. The membrane was washed again 6×5 min in PBST and then incubated with a 1 : 2000 dilution of streptavidin-horseradish peroxidase conjugate (Sigma) in PBST for 30 min at room temperature. Enhanced chemiluminescence (ECL) was performed according to the manufacturer's instructions (Amersham International, Amersham, UK) and the signal detected on Kodak XAR-5 film.

Western ligand blotting of IGFBP

Cells were plated onto 3.5-cm plastic tissue culture dishes in phenol red-free RPMI 1640 medium supplemented with 5% DCFCS with or without 5×10^{-5} M zinc chloride as required, at a density of 1×10^5 cells/ml. After 6 days, medium was removed, cells washed twice with phenol red-free RPMI 1640 medium and 1 ml serum-free medium (as above) added to each dish. After a further 24 h, conditioned medium was collected from each dish, centrifuged briefly to remove cell debris and stored at -70°C . Cells on each dish were counted on a Coulter counter as described above. Proteins were separated by 15% polyacrylamide SDS gel electrophoresis. To each gel track was loaded the volume of conditioned medium equivalent to 5×10^5 cells diluted $\times 2$ in gel sample buffer (26 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue). Proteins were transferred onto Hybond C Extra (Amersham International) by semi-dry Western blotting in 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol. Western blots were hybridized to ^{125}I -IGFI as described by Hossenlopp *et al.* (1986).

Measurement of IGF receptors

Cells were plated onto 24-well plastic tissue culture dishes in 0.5 ml aliquots in RPMI 1640 medium supplemented with 5% DCFCS at 0.5×10^5 cells per dish and grown for 5 days. Cells were then washed in RPMI 1640 medium and transferred into serum-free medium (RPMI 1640 medium supplemented with 15 mM HEPES buffer, 0.25% w/v bovine serum

albumin (BSA) (fraction V, Sigma)) and left overnight. After 24 h, three dishes of each treatment were used to estimate cell numbers by Coulter counting as above, and three dishes were used to assay IGFR by radioligand binding assay. For the binding assay, cells were washed twice with ice-cold phenol red-free RPMI 1640 medium and incubated with 80 000 cpm of ^{125}I -IGF-I (specific activity 7.4–9.25 TBq/mmol (200–250 Ci/mmol) made by the iodogen method (Salacinski *et al.* 1981)) with or without 30 μM insulin in 0.2 ml of phenol red-free RPMI 1640 medium containing 1 mg/ml BSA for 3 h at 4°C. After incubation, cells were washed three times with ice-cold isotonic saline, the cell layer was solubilized in ice-cold 0.5 M sodium hydroxide and incorporated radioactivity was measured in a gamma counter. Since insulin at 30 μM suppresses only receptor binding and does not bind to binding proteins (Conover 1992), specific receptor binding was assessed by subtraction of non-receptor binding in the presence of insulin from total binding in the absence of insulin. Results are expressed as disintegrations per minute (dpm) ^{125}I -IGFI bound per 10^6 cells.

Transient transfection assays using AP1-CAT

The *AP1-CAT* vector consisted of the the consensus *AP1* sequence inserted upstream of the thymidine kinase promoter and the coding sequence for chloramphenicol acetyl transferase (CAT) (from D. R. Soprano) (Soprano *et al.* 1996). Cells were grown in monolayer culture in 3.5-cm tissue culture dishes in phenol red-free DMEM with 5% DCFCS from a density of 2.0×10^5 cells per dish for 7 days. Cells were then transfected for 6 h with 5 μg of *AP1-CAT* per dish using the calcium phosphate precipitation method (Wigler *et al.* 1979). Cells were washed in phenol red-free DMEM, shocked with 25% glycerol in DMEM for 1 min, washed again and incubated overnight in phenol red-free RPMI1640 medium with 5% DCFCS. The following day, the medium was changed to 5% DCFCS in phenol red-free RPMI1640 containing the required concentration of oestradiol or zinc chloride. Cells were harvested 48 h later and assays for CAT activity performed as described previously on triplicate dishes (Sleigh 1986). Triplicate dishes of all treatments were also used for cell counting (see above) and results were normalized per 10^6 cells.

RESULTS

Overexpression of IGFII in ZR-75-1 cells.

Characterization of clones

The p Δ MT12 expression vector is an inducible expression vector utilizing the metal response elements of the human metallothionein IIA promoter linked to the coding region of human *prepro-IGFII*. Transfection of this vector into eucaryotic cells enables zinc-inducible overexpression of IGFII (Daly *et al.* 1991). ZR-75-1 cells were co-transfected with the p Δ MT12 expression vector and the pSV2neo selection plasmid, and stable G418 resistant clones were isolated. Clones were screened for overexpression of zinc-inducible *IGFII* mRNA by Northern blotting, and the results for three positive clones are shown in Figure 1. Clones designated ZRI3, ZRI12, and ZRI16 all produced enhanced basal levels of *IGFII* mRNA, compared with untransfected cells, and levels of *IGFII* mRNA were further increased by treatment of the cells with zinc. Western immunoblotting of the conditioned medium from these three clones confirmed that these cells also produced and secreted enhanced basal levels of IGFII protein compared with untransfected cells and IGFII protein was further increased by zinc treatment of the cells (Figure 2). Clones of cells were routinely maintained without zinc chloride, but after 8 days of zinc treatment, cells secreted a 22-kDa form of IGFII, a 15-kDa form of IGFII and small amounts of the mature 7 kDa IGFII (Fig. 2a). This is in line with the pattern of IGFII protein secretion by IGFII transfected MCF7 cells (MI7 cells)

Figure 1. Identification of G418-resistant clones of ZR-75-1 human breast cancer cells showing zinc-inducible overexpression of *IGFII* mRNA. Cells were co-transfected with pΔMT12 and pSV2neo and selected for resistance to G418 sulphate. Northern blot of whole cell RNA from untransfected ZR-75-1 cells (lanes 1,2) or three clones of G418-resistant cells ZRI3 (lanes 3,4), ZRI12 (lanes 5,6) ZRI16 (lanes 7,8) grown in monolayer culture for 7 days in the absence (–) or presence (+) of 5×10^{-5} M zinc chloride. The blot was probed for *IGFII* mRNA and equal loading of RNA samples was controlled by overprobing for the constitutively expressed *36B4* mRNA.

described previously (Lee *et al.* 1994). Long-term zinc treatment of the cells for 100 days resulted in secretion of similar levels of the 22 kDa and 7 kDa forms of IGFII but higher levels of the 15 kDa IGFII (Figure 2b compared with Figure 2a), and levels of all IGFII forms were reduced after 8 days of zinc withdrawal (Figure 2b lanes 12,14,16 compared with lanes 11,13,15). Treatment of the cells with zinc for 7 days but omission of zinc for the 24 h preparation period of conditioned medium resulted in reduced levels of the 22 kDa IGFII and increased levels of the mature 7 kDa IGFII (Figure 2c, lanes 21,23,25) compared with maintaining zinc throughout the 24 h conditioning period (Figure 2a, lanes 1,3,5). Levels of all zinc-inducible forms of secreted IGFII were never less in the ZRI3, ZRI12 and ZRI16 clones than in the equivalent MCF7 transfected MI7 cells (Figure 2c).

Growth response to overexpressed IGFII

Despite zinc-inducible overexpression of IGFII mRNA and protein, clones ZRI3, ZRI12 and ZRI16 showed no growth response to zinc treatment. When maintained in 5% DCFCS in the absence of phenol red, ZR-75-1 stock cells exhibit an oestrogen-dependent phenotype in monolayer culture such that cells proliferate exponentially in the presence of 10^{-8} M oestradiol but in the absence of oestradiol are limited to about one doubling (Glover, Irwin &

Figure 2. Secretion of IGFII protein by ZRI3, ZRI12 and ZRI16 cells. Western immunoblot of IGFII in conditioned media from untransfected ZR-75-1 cells (lanes 9,10), ZRI3 cells (lanes 1,2,11,12,21,22), ZRI12 cells (lanes 3,4,13,14,23,24), ZRI16 (lanes 5,6,15,16,25,26) and the previously transfected MI7 cells (lanes 18,19). Cells were grown as described in the *Materials and methods* section with the following variations: (a) for 7 days in the presence of 5×10^{-5} M zinc chloride, then maintaining the presence of zinc during the 24 h conditioned medium preparation; (b) for 100 days in the presence of 5×10^{-5} M zinc chloride, then maintaining the presence of zinc during the 24 h conditioned medium preparation; (c) for 7 days in the presence of 5×10^{-5} M zinc chloride, but then omitting zinc during the 24 h conditioned medium preparation. Conditioned medium was loaded in each lane equivalent to secreted proteins from 1.0×10^5 cells (a), 0.6×10^5 cells (b), or 0.3×10^5 cells (c). The position of mature IGFII (10 ng per lane) purchased commercially is shown in lane 8,20,27 as a positive control. Molecular weight markers (biotinylated from Amersham International) are shown in lanes 7,17,28.

Darbre 1988). Transfected clones ZRI3, ZRI12 and ZRI16 continued to display this phenotype and addition of 5×10^{-5} M zinc did not alter either basal or oestrogen-stimulated growth in any of the three clones (data not shown here except for effects on basal growth of ZRI16 cells in Figure 7a). This contrasted with previous results with parallel transfections into MCF7 cells, where the same concentration of zinc produced a substantial growth response in MI7 cells (Daly *et al.* 1991).

A range of experiments were then conducted in order to investigate whether any growth response to induced IGFII could be detected in any of the three clones by varying growth

conditions. Different concentrations of metal ions (0.25–100 μM zinc and 0.25–1.0 μM cadmium) were tested in line with concentrations shown previously to act on this inducible promoter system (Daly *et al.* 1991). Serum levels were reduced to 2.5% DCFCS and plating density of the cells was varied. None of these manipulations produced any growth response to metal ion induction of IGFII in any of the three clones (data not shown). In the event that prolonged exposure to zinc might sensitize the cells, ZRI3, ZRI12 and ZRI16 cells were grown for up to 200 days in the presence of 5×10^{-5} M zinc chloride, but even in the long-term treated cells, zinc had no effect on basal or oestrogen-stimulated cell growth (data not shown).

IGFII sensitivity of clones

Studies were performed to ascertain sensitivity of the transfected clones to IGFII, in order to determine whether the lack of growth response reflected an inability of the clones to respond to IGFII in general. Measurement of IGFR by radioligand binding assay showed that all three clones possessed receptors capable of binding ^{125}I -IGFI (4562 ± 1016 dpm bound per 10^6 cells (ZRI3); 3616 ± 319 dpm bound per 10^6 cells (ZRI12); 5517 ± 166 dpm bound per 10^6 cells (ZRI16)) as did the untransfected parental ZR-75-1 cells (2840 ± 80 dpm bound per 10^6 cells). Furthermore, Figure 3 shows that clones ZRI3, ZRI12 and ZRI16 could all respond to exogenously added IGFII. Growth was increased in all three clones as well as in the untransfected cells in a dose-dependent manner at concentrations of IGFII ranging from 10 to 100 ng/ml.

Figure 3. Effects of exogenous IGFII on the growth of ZRI3, ZRI12 and ZRI16 cells as compared to untransfected ZR-75-1 cells in monolayer culture. Plating density of the cells is given at day 0 for each separate experiment (open bar). Cells were then grown for 14 days in RPMI1640 medium with 5% DCFCS with no further addition (bar with horizontal lines) with 10 ng/ml IGFII (bar with wide sloping lines), with 100 ng/ml IGFII (bar with narrow sloping lines), or with 10^{-8} M oestradiol (black bar). Error bars represent the standard error of triplicate dishes, and where not seen, the SE was too small to be shown.

IGFBP in clones

Since IGFBP are known to inhibit IGF-stimulated growth by sequestering the ligand (Kelley *et al.* 1996), the lack of growth response to zinc could be explained by increased secretion of IGFBP. However, Western ligand blotting of secreted IGFBP from ZRI3, ZRI12 and ZRI16 cells in the absence or presence of 5×10^{-5} M zinc chloride did not reveal any increase in IGFBPs relative to either control untransfected ZR-75-1 cells or MCF7 transfected MI7 cells (data not shown).

Bioactivity of overexpressed IGFII

In order to determine whether the zinc-induced IGFII from the clones was bioactive, conditioned media from the clones were tested for their growth promoting ability. Figure 4 shows that medium conditioned in the presence of 5×10^{-5} M zinc chloride by ZRI3, ZRI12 and ZRI16 cells was able to stimulate growth of untransfected MCF7 cells whereas control medium conditioned by untransfected ZR-75-1 cells had no effect on MCF7 cell growth. This suggested that the zinc-induced IGFII secreted by ZRI3, ZRI12 and ZRI16 cells was bioactive on MCF7 cell growth.

Since the clones secreted bioactive IGFII and were intrinsically capable of responding to IGFII, effects of zinc were then examined on two molecular markers known to be influenced by IGFII in breast cancer cells. It is now known that exogenous IGFII can increase expression of the oestrogen-regulated gene *pS2* (Chalbos *et al.* 1993) and that autocrine IGFII is even more effective in this action in MI7 cells (Perachiotti & Darbre 1995). Figure 5a shows that in ZRI16 cells, *pS2* mRNA levels were increased not only by

Figure 4. Effects on the growth of untransfected MCF7 cells in monolayer culture by medium conditioned with untransfected ZR-75-1 cells, ZRI3 cells, ZRI12 cells or ZRI16 cells. Plating density of the MCF7 cells is given at day 0 for each separate experiment (open bar). MCF7 cells were then grown for 14 days in RPMI 1640 medium with 5% DCFCS (bar with horizontal lines) or in the same medium but which had been conditioned for 24 h previously by untransfected ZR-75-1 cells, ZRI3 cells, ZRI12 cells or ZRI16 cells (black bar). Errors represent the standard error of triplicate dishes, and where not seen, the SE was too small to be shown.

Figure 5. Zinc regulation of *pS2* mRNA expression (a) and AP1-CAT activity (b) in ZR16 cells in monolayer culture. (a) Northern blot of whole cell RNA from ZR16 cells grown in monolayer culture for 7 days in RPMI1640 medium with 5% DCFCS followed by a further 24 h (lanes 1–3) or 6 days (lanes 4–6) in the same medium alone (–) (lanes 3,6) or with 10^{-8} M oestradiol (+E) (lanes 1,4) or with 5×10^{-5} M zinc chloride (+Zn) (lanes 2,5). The blot was probed for *IGFII* mRNA and equal loading of RNA samples was controlled by overprobing for the constitutively expressed 36B4 mRNA. (b) ZR16 cells were grown in monolayer culture for 7 days in DMEM with 5% DCFCS, transiently transfected for 6 h with AP1-CAT DNA and then grown for 48 h in DMEM/5% DCFCS alone (–), with 5×10^{-5} M zinc chloride (+Zn), with 100 ng/ml IGFII (+IGFII) or with 10^{-8} M oestradiol (+E). CAT activity is expressed as cpm of 14 C-acetyl group transferred from 14 C-acetyl CoA to chloramphenicol per hour per 10^6 cells.

exposure to oestradiol but also following a 24-h exposure to zinc. Since IGFs are capable of increasing expression of AP1-regulated genes (Monnier *et al.* 1994), the ability of the zinc-induced IGFII to influence *AP1* activity was investigated in the clones. Figure 5b shows that transient transfection of a synthetic *AP1-CAT* gene into ZR16 cells resulted in increased levels of CAT activity not only by exogenous IGFII and oestradiol but also by 5×10^{-5} M zinc chloride.

Overexpression of IGFII and PC2 in ZR-75-1 cells

Since Figure 2 shows that ZR13, ZR112 and ZR116 cells all secreted large amounts of 22 kDa and 15 kDa forms of IGFII as compared to the MI7 cells, experiments were performed to determine whether reduction in levels of these large forms of IGFII could restore a growth response in the ZR-75-1-derived clones. ZR116 cells were transfected with the pSR α /PC2 expression vector to constitutively overexpress the protein processing gene *PC2*. Stably transfected clones were selected by hygromycin-B-resistance and screened for increased processing of zinc-induced IGFII by Western immunoblotting. Six clones designated ZR116-P1–ZR116-P6 all secreted reduced levels of the 22 kDa IGFII and slightly more of the mature 7 kDa IGFII compared with ZR116 cells (Figure 6). Despite reduced levels of secreted 22 kDa IGFII protein, none of these six clones showed any growth response to zinc. Zinc did not alter either basal or oestrogen-stimulated growth in 5% DCFCS over 14 days in any of the six clones (data not shown).

Figure 6. Secretion of IGFII by ZRI16 cells transfected with genes to ensure simultaneous overexpression of both IGFII and the protein processing enzyme PC2. ZRI16 cells were co-transfected with plasmids pSR/PC2 and pREP4, and clones of hygromycin-resistant cells isolated. An immunoblot was then prepared of IGFII secreted in the conditioned medium from each of six hygromycin-resistant clones (designated P1-P6) and ZRI16 cells. Cells were grown for 7 days in the presence of 5×10^{-5} M zinc chloride and hygromycin and conditioned medium prepared for 24 h in the continued presence of zinc chloride. Conditioned medium was loaded in each track equivalent to secreted proteins from 0.5×10^5 cells. Molecular weight markers (biotinylated from Amersham International) are shown in lane 8.

Overexpression of IGFII and IGFIR in ZR-75-1 cells

Although the ZRI3, ZRI12 and ZRI16 clones possessed receptors capable of binding 125 I-IGFI, levels were of the order of one-third to one-half (see above) of those found in the MI7 cells (11072 ± 41 dpm bound per 10^6 cells). In order to determine whether the lack of growth response to zinc-inducible IGFII resided in the lower levels of IGFR, ZRI16 cells were transfected with the pCMV5 expression vector to constitutively overexpress IGFIR. Stably transfected clones were selected by hygromycin-B-resistance and assayed for levels of receptor overexpression by 125 I-IGFI binding assay. Clone ZRI16-R4 produced levels of IGFR increased by 3.1-fold relative to ZRI16 cells. Figure 7 shows that, unlike the ZRI16 cells, the ZRI16-R4 cells showed a growth response to zinc. Growth experiments in 5% DCFCS over 14 days showed that 5×10^{-5} M zinc resulted in greatly increased growth of ZRI16-R4 cells to a level near to that of 10^{-8} M oestradiol.

DISCUSSION

This report describes the effects of overexpression of IGFII within the oestrogen-dependent ZR-75-1 human breast cancer cell line. Previous work has shown that overexpression of IGFII in MCF7 cells could result in increased basal cell growth in monolayer culture, increased anchorage-independent growth and reduced oestrogen sensitivity of cell growth (Daly *et al.* 1991; Cullen *et al.* 1992). In the MI7 clone of transfected cells, zinc-inducible *IGFII* mRNA (Daly *et al.* 1991) and protein (Lee *et al.* 1994) increased cell growth via an autocrine loop which could be inhibited by blockade of the IGFIR (Daly *et al.* 1991). Studies described here demonstrate that parallel transfections into the ZR-75-1 cell line resulted in three clones of cells in which there was zinc-inducible overexpression of *IGFII* mRNA and secreted protein to a similar extent as in MI7 cells and yet which showed no growth response to zinc. The clones all possessed receptors for IGF, were capable of responding to exogenously added IGFII and did not overproduce growth inhibitory IGFBP. The secreted

Figure 7. Growth response to zinc and oestradiol for ZRI16 cells (ZR-75-1 cells transfected with *IGFII*) and ZRI16-R4 cells (ZR-75-1 cells transfected with *IGFII* and *IGFIR*) in monolayer culture. Cells were grown for up to 14 days in RPMI1640 medium with 5% DCFCS with no further addition (○), with 5×10^{-5} M zinc chloride (□) or with 10^{-8} M oestradiol (●). Bars represent the standard error of triplicate dishes, and where not seen, the SE was too small to be shown.

IGFII was bioactive in being able to increase the growth of MCF7 cells, and in being able to induce molecular responses of *pS2* and *API-CAT* gene expression in the ZR-75-1 cells themselves. The reason for the lack of growth response to zinc in the transfected ZR-75-1 cells appeared to reside in the level of receptors for IGF, since a growth response to zinc-induced IGFII could be found when there was simultaneous overexpression of IGFIR. Transfection of an *IGFIR* gene in conjunction with the *IGFII* gene into ZR-75-1 cells needed to increase IGFR levels by only threefold in order for the zinc-induced IGFII to produce increased cell growth and reduced oestrogen sensitivity for growth. Interestingly, this order of a threefold increase raises levels of IGFR in the ZR-75-1 cells to those found endogenously in the MCF7 cells (see *Results* section).

These results emphasize the importance of the delicate balance between ligand and receptor in growth factor interactions on breast cancer cell growth. The data suggest that proliferative response to IGFII in breast cancer cells is determined by a combination of both receptor number and receptor occupancy. The lack of ZR-75-1 growth response to transfected *IGFII* did not result merely from inadequate receptor numbers which were saturated at low ligand levels because growth response could be generated by addition of exogenous IGFII as well as by overexpression of receptor. This implies that the proliferative effects can result from either increasing receptor occupancy with more ligand or increasing receptor number without altering occupancy. Since the IGFIR can signal through multiple pathways (Surmacz *et al.* 1998), it remains to be determined how receptor number and receptor occupancy might affect the signalling pathway employed for IGFII in breast cancer cells.

Another interesting feature of IGFII action in the ZR-75-1 breast cancer cells was that limiting levels of receptor appeared to be different for different responses. Molecular responses appeared to require lower levels of receptor than cell growth responses, in that zinc-induced overexpression of IGFII could stimulate gene expression systems using intrinsic

levels of IGF receptors but effects on cell growth required overexpression of IGFIR. This fits with other data suggesting that the threshold of oestradiol required to induce progesterone receptor (PR) expression in normal breast epithelial cells is lower than that required to induce proliferation (Clarke, Howell & Anderson 1997), and that in rainbow trout, two oestrogen-regulated genes can have different oestrogen receptor level requirements for transcriptional response (Flouriot *et al.* 1997). This implies a heterogeneity in limiting requirements for different types of responses and could even suggest multiple signalling pathways with different receptor thresholds. It also illustrates the danger in using molecular markers to predict proliferative response.

The emphasis of the importance of cell context in overexpression studies aids interpretation of previously published work where IGFII (Daly *et al.* 1991, Cullen *et al.* 1992), heregulin- β 2 (Tang *et al.* 1996), FGF4 (McLeskey *et al.* 1993) and TGF β 1 (Arteaga *et al.* 1993) could all disrupt growth of tested breast cancer cells when overexpressed, but TGF α (Clarke *et al.* 1989) could not. This has remained in marked contrast to clinical studies demonstrating relevance of TGF α levels to clinical outcome (Nicholson *et al.* 1995; Miller 1996). It can now be suggested that TGF α was no less important to breast cancer cell growth *in vitro* than the other growth factors but rather that the lack of growth response was probably a result of some other limiting component in the signal transduction pathway for TGF α in those cells tested. For the other growth factors in the cell lines tested, ligand alone was the limiting factor and overexpression of ligand was therefore sufficient to deregulate growth. Lack of effect on cell growth with EGFR (Van Agthoven *et al.* 1992) and IGFR (Daws *et al.* 1996, Guvakova & Surmacz 1997) overexpression implies that in the cell lines tested, those receptors were again not limiting and some other component (either ligand itself or some other signal transduction component) was probably the limiting factor. The concept of rate-limiting steps has been well recognized within metabolic pathways for several decades but such points have yet to be clearly identified in signal transduction pathways. It would seem intrinsically logical for cells to have key rate-limiting steps within signal transduction pathways and experiments in other systems have suggested that receptor levels can be rate limiting in IGF action in mouse embryo cells (Kaleko, Rutter & Miller 1990), haematopoietic cells (McCubrey *et al.* 1991) and fibroblasts (Rubini *et al.* 1997), in nerve growth factor action in neuroblastoma cells (Azar *et al.* 1994), in T cell antigen signalling (Itoh & Germain 1997) and in EGF signalling in rat kidney fibroblasts (Lahaye *et al.* 1998). By contrast, however, some cells seem unable to tolerate excessively high levels of receptor in that overexpression of some receptors can reverse responses from growth stimulatory to growth inhibitory (McLeskey *et al.* 1994). This would imply that receptor levels need to remain within certain limits, such that if they fall too low the response disappears, but if they rise too high detrimental consequences can follow.

These results may also help to explain the problems in identifying single growth factor markers with prognostic significance for tumour response in patients. A substantial body of evidence implicates the IGF system as a key regulator of breast cancer cell growth *in vivo* as well as *in vitro*. IGFII under the control of the β -lactoglobulin regulatory element can induce breast tumours in transgenic mice (Bates *et al.* 1995), and breast tumour xenografts have been inhibited by anti-IGF strategies (Yee 1994). In clinical material, IGFIR has been found overexpressed in many human breast cancers, and generally low levels of IGFIR or high levels of IGFBNs are suggested to be poor prognostic indicators (Lee, Hilsenbeck & Yee 1998). Correlations with IGFII levels are less clear, maybe owing to its localization in stromal cells adjacent to the cancer rather than always in the malignant epithelial cells (Giani *et al.* 1998; Rasmussen & Cullen 1998). Review of the clinical literature (Lee *et al.* 1998) suggests,

in accordance with the present studies here, that clinical response may be better predicted from measurement of several, rather than one, components of the IGF system. This has already been well acknowledged for oestrogen receptor and progesterone receptor analysis in breast tumours where the presence of progesterone receptor as a functional marker of oestrogen receptor enhances the likelihood of response to endocrine therapy (Miller 1996). Clearly, a thorough kinetic analysis of the IGF system is now needed in order to provide more specific information on key limiting steps within breast cancer cells and on relative levels of key interacting components required for proliferative response.

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