Altered expression of the IGF-1 receptor in a tamoxifen-resistant human breast cancer cell line

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Summary The relationship between oestrogen (E_2) and insulin-like growth factor-one (IGF-1) was examined in both tamoxifen-sensitive (MCF 7/5–21) and tamoxifen-resistant (MCF 7/5–23) subclones of the MCF 7 cell line. Both subclones were grown in defined, serum-free (SF) medium over a period of 7 days with the addition of E_2 or IGF-1 or a combination of both agents. Growth of both MCF 7/5–21 and 7/5–23 cells was stimulated (245% and 350%, respectively) by E_2. However, only the growth of MCF 7/5–23 cells was stimulated (266%) by IGF-1. A combination of E_2 and IGF-1 significantly enhanced MCF 7/5–21 and 7/5–23 cell growth (581% and 695%, respectively). E_2-induced IGF-1 receptor (IGF-1R) levels (as measured by ^{125}I-IGF-1 binding and Northern analyses) in only MCF 7/5–23 cells. This effect was partially inhibited by tamoxifen. In medium containing serum, the growth of only the MCF 7/5–23 cells was significantly inhibited by the IGF-1R monoclonal antibody, αIR-3. The detection of E_2-induced expression of IGF-2 using RT-PCR was demonstrated in the MCF 7/5–23 cells. These experiments indicate that E_2 may sensitize tamoxifen-resistant MCF 7/5–23 cells to the growth stimulatory actions of IGF-2 via up-regulation of the IGF-1R and describes a cell-survival mechanism that may manifest itself as tamoxifen resistance.

Keywords: breast cancer; IGF-1R; oestrogen; tamoxifen resistance

Oestrogens (E_2) stimulate the proliferation of human breast cancer cells predominantly via the oestrogen receptor (ER). However, the clinical effectiveness of antioestrogens such as tamoxifen in ER+ cells is occasionally limited by intrinsic resistance or, more commonly, the acquisition of resistance. The mechanisms underlying the development of anti-oestrogen insensitivity remain elusive, particularly as it is now recognized that tamoxifen resistance is generally not associated with the loss or abnormal function of the ER (Encarnacion and Fuqua, 1994). An alternative mechanism may be the progression of breast tumours to a hormone-independent proliferative state whereby growth factors act as the principal mitogens (Dickson et al, 1993). Of particular interest is the insulin-like growth factor (IGF) family. IGF-1 is a mitogen for human breast cancer cells in vitro (Lippman, 1985), especially in the presence of E_2, and acts primarily via a specific cell surface glycoprotein receptor known as the type 1 IGF receptor (IGF-1R; Steele-Perkins et al, 1988).

Evidence for the role of IGF-1 in breast cancer has come from case-control studies that have reported increased circulating levels of IGF-1 in women presenting with breast cancer (Peyrat et al, 1993). Furthermore, the expression of IGF-1R mRNA has been found in most breast cancer cell lines and in over 90% of human breast tumour specimens (Papa et al, 1993). Pekonen et al (1988) have demonstrated a positive correlation between IGF-1R expression and ER and progesterone receptor (PR) content and noted an increase in expression in malignant compared with adjacent normal tissue. Finally, the majority of recent reports have demonstrated that expression of the IGF-1R is vital for the inhibition of apoptosis in tumour cells (Resnicoff et al, 1995; Dunn et al, 1997). It is now widely postulated that IGF-1R signalling is probably more important in the role of tumour cell survival and protection from apoptosis than in mitogenesis (Baserga et al, 1997).

There is also mounting evidence for an interaction between the IGF-1R and ER signalling pathways (Westley and May, 1994). Although IGF-1 can stimulate the proliferation of E_2-responsive breast cancer cells on its own, in the presence of E_2 a marked synergistic effect is usually observed. This has been associated with the observation that E_2 can cause changes in the expression of the IGF-1R and/or its ligands (Stewart et al, 1990; Lee and Yee, 1995). Conversely, it has been shown that IGF-1 can influence the function of the ER. A characteristic response to E_2 is the up-regulation of PR expression. However, physiological concentrations of IGF-1 were also found to increase the PR level in MCF 7 breast cancer cells (Katzenellenbogen and Norman, 1990). This stimulation was blocked by an anti-oestrogen suggesting that IGF-1 was acting on a component of the ER pathway. In the absence of any E_2, Newton et al (1994) also observed stimulation of ER-mediated reporter-gene activity by IGF-1 in the pituitary tumour cell line, GH$_3$. The mechanism of this ligand-independent activation of the ER is still unclear but it may be involved in the development of tamoxifen resistance.

In this study, we have evaluated the comparative effects of both E_2 and IGF-1 on cell growth in an in vitro model of intrinsic tamoxifen-resistant, ER+ breast cancer in defined, serum-free conditions. Our results suggest that the tamoxifen-resistance exhibited by the MCF 7/5–23 cells (relative to the tamoxifen-sensitive MCF 7/5–21 cells) may be associated with IGF-mediated cell proliferation.

MATERIALS AND METHODS

Reagents

Oestradiol and tamoxifen were purchased from Sigma Chemicals (St Louis, MO, USA). Human recombinant IGF-1 was purchased from GroPep (Adelaide, Australia). Oestradiol and tamoxifen were dissolved in absolute ethanol whereas IGF-1 was dissolved in 10 mM hydrochloric acid (HCl) with 0.1% bovine serum albumin (BSA) (essential fatty acid-free; Sigma Chemicals). ^{125}I-IGF-1 (S.A: 2800 Ci mmol^{-1}) was purchased from NEN-DuPont (Sydney, Australia). Monoclonal antibody to IGF-1R (αIR-3) was obtained from Oncogene Science (Cambridge, MA, USA).

Cell culture

Two cell lines were used in these studies; a tamoxifen-sensitive (MCF 7/5–21) and a tamoxifen-resistant (MCF 7/5–23) subclone of the parental, sensitive MCF 7 line. The tamoxifen resistance was previously characterized as a 22-fold relative difference (Hu et al, 1993) in the concentration of tamoxifen required to inhibit cell growth by 50% in a growth assay performed in medium containing serum. This characteristic phenotype was also confirmed for the current study. Both subclones express the oestrogen-receptor and were repeatedly found to be *Mycoplasma* free. Stock cultures were maintained in Roswell Park Memorial Institute (RPMI)-1640 (ICN Biomedicals, Aurora, OH, USA) supplemented with 10% fetal bovine serum (FBS: Cytosystems, Sydney, Australia), 6 mM L-glutamine (CSL, Melbourne, Australia), 0.112% NaHCO$_3$ (ICN Biomedicals), 20 mm Hepes (CSL), 10 μg ml^{-1} human insulin (Actrapid HM, Novo Nordisk, Sydney, Australia) and 20 μg ml^{-1} gentamicin (David Bull Laboratories, Melbourne, Australia) at 37°C in a humidified chamber containing 5% carbon dioxide (CO$_2$).

Cell growth assays: stock (serum-containing) conditions

The relative tamoxifen-resistant growth of the MCF 7/5–23 cells as compared to the tamoxifen-sensitive MCF 7/5–21 cells in stock medium was confirmed using dose–response assays that have been previously described (Hu et al, 1993). Cell growth experiments were also performed in stock medium with the IGF-1R mono-clonal antibody (mAb), αIR-3 (Kull et al, 1983). Cells were seeded in 12-well plates (Costar) at a concentration of 1×10^4 cells/well in 1 ml of stock medium. Forty-eight hours later, fresh stock medium containing αIR-3 (1 μg ml^{-1}) or vehicle (1 μg ml^{-1} mouse IgG$_1$) was added and the cells were further incubated for 5 days. The rate of cell proliferation was determined by using the MTT colorimetric cell growth assay kit (Sigma Chemicals) according to the manufacturer's specifications which involved spectrophotometric measurement of cell growth as a function of the mitochondrial activity of living cells.

Serum-free conditions

A week before each experiment, cells were withdrawn from endogenous steroids present in FBS as originally described by Musgrove and Sutherland (1993). Stock cell cultures were passaged into phenol red-free RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) containing all the supplements described above with the exception of FBS which was substituted with 10% dextran–charcoal-treated FBS (DCC medium; Darbre et al, 1983). The DCC medium was replenished twice over the next 5–7 days by which time cells had reached exponential growth phase and were ready for experimental use.

To assess the effect of E$_2$ or IGF-1, as well as E$_2$ in combination with IGF-1, on growth rates, cells were seeded in 6-well plates (Costar, Cambridge, MA, USA) at an initial concentration of 1×10^5 cells/well in 2 ml of DCC–FBS medium. Twenty-four hours later, the medium was changed to 5 ml of serum-free, phenol red-free RPMI-1640 supplemented with transferrin (30 nM; Boehringer, Mannheim, Germany), 6 mM L-glutamine, 0.112% NaHCO$_3$, 20 mM Hepes and 20 μg ml^{-1} gentamicin (SF medium) and 10 μg ml^{-1} human insulin. A further 24 h later, SF medium without insulin but containing 1.0 nM of IGF-1, E$_2$ or combinations thereof or equivalent volumes of vehicle solvent were added to the cells as stated in the figure legends. Cell numbers were determined daily after the cells were harvested with a 0.05% trypsin (CSL) and 0.02% EDTA (Flow) solution and viable cells (based on trypan blue dye exclusion) counted using a haemocytometer. All experiments were performed in triplicate and were repeated at least once. Results obtained at the varying concentrations of each mitogen were expressed as a percentage of vehicle control, the mean ± standard error of the mean (SEM).

IGF-1 binding assays

To determine the role of E$_2$ in up-regulating the IGF-1R, ^{125}I-IGF-1 binding was measured in monolayers of MCF 7/5–21 and MCF 7/5–23 cells that had been progressively withdrawn from medium containing serum in an identical manner as for the cell growth assays. Prior to the binding assay, the cells were seeded into 25 cm^2 culture flasks (Corning Inc., Corning, NY, USA) in DCC medium at a density of 10^6 cells/flask. After allowing 24 h for cell attachment, the medium was changed to SF medium with insulin. Twenty-four hours later, cells were washed once with sterile phosphate-buffered suline (PBS) and incubated in SF medium containing vehicle, E$_2$, tamoxifen or a combination of E$_2$ and tamoxifen, as stated in the figure legends. Cells were incubated (in quadruplicate) with these reagents for a period of 3–5 days. The monolayers were then washed once with binding assay buffer; sterile PBS containing 0.1% essential fatty acid-free BSA and incubated with 10–15 pM ^{125}I-IGF-1 in 5 ml of binding assay buffer for up to 16 h at 4°C.

Non-specific binding was determined by incubating cells with ^{125}I-IGF-1 in the presence of 100 nM unlabelled IGF-1 and 50 μM insulin and was typically 10–20% of total bound radioactivity. Specific binding in the presence of either αIR-3 (10 μg ml^{-1}) or insulin (50 μM) was also determined to estimate the proportion of total ^{125}I-IGF-1 binding to the IGF-1R or to IGF binding proteins (IGFBP). These experiments utilized the distinctive binding properties of the αIR-3 antibody and insulin, both of which will effectively compete for binding with ^{125}I-IGF-1 to the IGF-1R at high concentrations but not to the IGFBP (Jones and Clemmons, 1995).

After the incubation period, the cells were washed three times with ice-cold binding assay buffer and solubilized with a 0.1% Triton X100, 0.5 M NaOH solution for subsequent determination of bound radioligand using a gamma counter (Cobra-II, Packard, Meriden, CT, USA) with an efficiency of 81% for detecting the ^{125}I isotope. The statistical significance of the data was established using the analysis of variance method followed by Fisher's multi-comparison test.

Northern analysis

Total RNA was isolated by acid guanidium thiocyanate/phenol-chloroform extraction (Chomczynski and Sacchi, 1987) of cells that were incubated with test reagents after a week of steroid deprivation as described above. All RNA concentrations were determined spectrophotometrically and the integrity of the RNA was visualized by electrophoretical separation on 1.25% agarose (IBI, New Haven, CT, USA)/2.2 M formaldehyde denaturing gels. RNA was transferred to nylon membranes (MSI, Westboro, MA, USA) by capillary action, UV fixed and stored at –20°C. Human (h)-IGF-1, h-IGF-2 or rat-IGF-1R cDNA probes (generous gifts from Dr Kay Lund) or 18S ribosomal RNA probes (Bresatec, Adelaide, Australia) were labelled using α-^{32}P-dCTP (NEN-DuPont) by random priming and hybridization was performed in a 50% formamide, 5 × SSPE [0.75 M potassium chloride (NaCl), 50 mM NaH$_2$PO$_4$·H$_2$O, 5 mM EDTA], 0.5% sodium dodecyl sulphate (SDS), 0.35 mg ml^{-1} herring sperm DNA and 5 × Denhardt's solution at 42°C for approximately 16 h. Blots were then washed in a 2 × SSC (0.3 M NaCl, 0.03 M Na$_3$citrate)/0.1% SDS solution for 10 min at 42°C, 1 × SSC/0.1% SDS for 15 min at 42°C and finally in 0.1 × SSC/0.1% SDS for 30 min at 55°C. Blots were exposed to X-ray film (NEN-DuPont) with intensifying screens at –70°C for 3–10 days. Quantification of radiolabelled bands was performed with the Instant Imager system (Packard, Meriden, CT, USA).

RT-PCR analysis

Poly (A)$^+$ RNA was isolated using the Quickprep Micro mRNA purification kit (Amrad Pharmacia, Sydney, Australia) from cells that were incubated with the reagents indicated in the figure legends under SF conditions. For cDNA synthesis, no more than 100 ng of poly (A)$^+$ RNA was added to 100 ng of random hexamers and double-distilled water (DDW) to a reaction volume of 10 μl. The mixture was incubated at 70°C for 5 min, chilled on ice for 2 min, followed by the addition of 10 μl of the cDNA synthesis master-mix such that the final reactions contained 50 mM Tris-HCl (pH 8.3), 1 mM each of dATP, dGTP, dCTP and dTTP (dNTP), 75 mM KCl, 3 mM magnesium chloride (MgCl$_2$), 5 mM dithiothreitol, 200 units of M-MLV reverse transcriptase (RT) (Gibco-BRL) and 10 units of RNase inhibitor (Gibco-BRL). The reaction mix was incubated at 37°C for 60 min, diluted with 30 μl of DDW and terminated by a final 5 min incubation at 90°C.

Both IGF-2 and histone 3.3 (H3.3) mRNA expression were assayed by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers for amplification. The IGF-2 primers were sense GGCTTCTACTTCAGCAGGC (exon 6) and antisense GTGGGTAGAGCAATCAGGG (exon 8) yielding a 344 bp product. The H3.3 primers were sense CCACT-GAACTTCTGATTCGC (exon 2) and antisense GCGTGC-TAGCTGGATGTCTT (exon 3) yielding a 214 bp product. The ubitiquously expressed H3.3 gene was used as an endogenous internal control for IGF-2 in the RT-PCR assay to check RNA integrity and loading. For IGF-2 detection, one-tenth of the reaction volume obtained after cDNA synthesis (5 μl) was diluted with a PCR-mixture (up to a final volume of 50 μl) such that the final reaction consisted of 0.5 μM each of the primer pair, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 mg ml^{-1} BSA, 1.5 mM MgCl$_2$, 0.2 mM of each dNTP and 1.0 unit of *Taq* polymerase (Promega, Sydney, Australia). The PCR mixture was overlaid with 50 μl of mineral oil (Sigma) to prevent evaporation and then underwent a

thermal cycling protocol consisting of 35 cycles of template denaturation at 95°C for 1 min, primer-template annealing at 57°C for 1 min and polymerase extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min on an Omnigene thermal cycler (Hybaid, Melbourne, Australia).

The H3.3 gene was always assayed simultaneously using the identical protocol as described above for the IGF-2 gene with the only exception being that the cDNA template was diluted 1:10 before PCR cycling due to the relatively high abundance of H3.3 expression compared to IGF-2. The PCR cycling parameters were determined from preliminary experiments where we demonstrated that amplification of both IGF-2 and H3.3 levels was in the exponential phase of PCR at 35 cycles for the concentrations of template that were used. Thus, levels of IGF-2 expression detected by RT-PCR were normalized for levels of H3.3 for every sample and comparisons of the IGF-2/H3.3 ratios between samples were made only within the same assay. The negative cDNA template control (DDW) was subjected to 40 cycles of PCR with no amplification of H3.3 or IGF-2 detected. Quantification of IGF-2 and H3.3 levels were performed using Molecular Analyst software on a UV-Gel-Documentation instrument (BioRad, Sydney, Australia) analysing the PCR product signal after size-fractionation on a 1.5% agarose gel.

RESULTS

Tamoxifen-sensitive and resistant breast cancer cells are E$_2$-responsive but differ in IGF-1 sensitivity

In the presence of tamoxifen, the proliferation of both MCF 7 subclones was significantly altered over a time-course of 7 days. In dose–response growth assays, the concentrations of tamoxifen that resulted in a 50% inhibition of cell growth (IC$_{50}$) were 66.1 nM and 1.08 μM in the MCF 7/5–21 and 7/5–23 subclones (Figure 1), respectively. This represented a 17-fold difference in sensitivity to tamoxifen, results that were similar to our earlier studies (Hu et al, 1993) which also demonstrated that the ER number and affinity of binding to E$_2$ was not different between the two cell lines (Parisot et al, 1995).

Both subclones were successfully cultured in defined, serum-free conditions after approximately a week of serum withdrawal. Under these conditions, the response to the mitogens E$_2$ and IGF-1 could be determined with negligible interference from residual E$_2$ and/or growth factors normally present in serum. By day 7 of cell culture, the growth of the tamoxifen-sensitive, MCF 7/5–21 cells was stimulated 2.5-fold by E$_2$ (compared to vehicle controls) at a physiological concentration of 1 nM (Table 1). The ethanol vehicle had a minimal effect on cell growth (data not shown). By itself, IGF-1 (1 nM) had no effect on the growth of these cells compared to vehicle controls; however, the combination of E$_2$ and IGF-1 markedly enhanced cell growth almost sixfold.

The growth of MCF 7/5–23 cells was stimulated 3.5-fold by E$_2$ by day 7 of the experiment. When E$_2$ and IGF-1 were combined, enhanced proliferation of MCF 7/5–23 cells was also demonstrated (almost sevenfold) compared with either agent alone (Table 1). In contrast to MCF 7/5–21 cells, IGF-1 alone enhanced the growth of MCF 7/5–23 cells to levels similar to those attained with E$_2$. A more detailed investigation of the response of both MCF 7/5–21 and MCF 7/5–23 cells to different doses of IGF-1 (up to 100 nM) over a 7-day period demonstrated that only the proliferation of the tamoxifen-resistant, MCF 7/5–23 cells increased in a

Table 1 Effect of E_2, IGF-1 or a combination of both agents on MCF 7/5–21 and MCF 7/5–23 cell proliferation

Cell line	E_2	IGF-1	E_2 + IGF-1
MCF 7/5–21[a]	245 ± 27[b]	119 ± 7	581 ± 26[b]
MCF 7/5–23[a]	350 ± 51[b]	266 ± 19[b]	695 ± 82[b]

Cells were plated in 6-well plates after being withdrawn from oestrogens as described in Materials and Methods. Triplicate wells were then treated for 7 days with SF medium supplemented with either vehicle (0.1% ethanol), E_2 (10^{-9} M), IGF-1 (10^{-9} M) or a combination of E_2 + IGF-1 (10^{-9} M). Cell numbers were measured and results were expressed as a % of the number of viable cells counted in the absence of any drug (vehicle only), with each number representing the mean ± SEM of three separate experiments. [a]Expressed as a % of vehicle control. [b]$P < 0.05$ by ANOVA and Fisher's multi-comparison statistical significance tests.
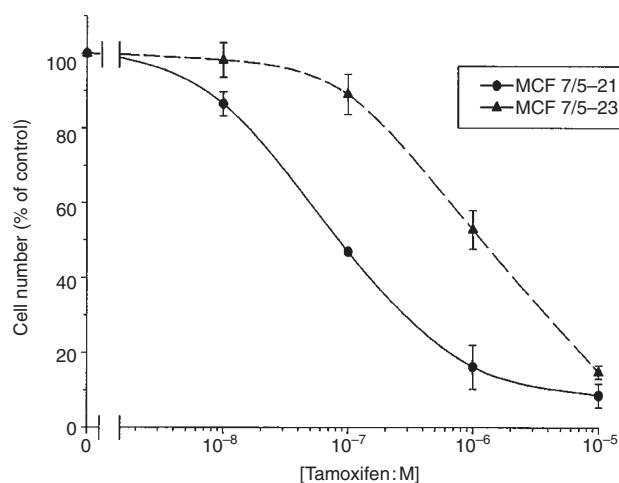

Figure 1 The effect of tamoxifen on MCF 7/5–21 and 7/5–23 cell proliferation. Cells from both subclones were seeded in stock medium and allowed to reach the exponential growth phase (48 h). The subclones were incubated with stock medium supplemented with either vehicle (0.1% ethanol) or a range of tamoxifen concentrations (10^{-8}–10^{-5} M) for a further 5 days as described in Materials and methods. Results are expressed as a % of the number of viable cells counted in the absence of any drug (vehicle only) with each bar representing the mean ± SEM of three separate experiments, each performed in triplicate

dose-dependent fashion (data not shown). Furthermore, tamoxifen had only a slight inhibitory effect on the IGF-1-mediated growth of MCF 7/5–23 cells (data not shown).

E_2 up-regulates IGF-1 binding in tamoxifen-resistant but not in tamoxifen-sensitive breast cancer cells

To determine whether the enhanced stimulatory effect of the combination of E_2 and IGF-1 on the growth of both MCF 7/5–23 cells and MCF 7/5–21 cells could be attributed to an alteration in the expression of the IGF-1R by E_2, we measured the effects of E_2, tamoxifen alone and E_2 plus tamoxifen on ^{125}I-IGF-1 binding. A twofold increase in the specific binding of ^{125}I-IGF-1 was demonstrated in the MCF 7/5–23 cell line after exposure to 1 nM E_2 (Figure 2A). A similar effect was not observed in the MCF 7/5–21 cell line (Figure 2A). Whereas, tamoxifen alone had no effect on the level of ^{125}I-IGF-1 binding in either cell line, this antioestrogen was able to partially inhibit the effect of 1 nM E_2 on the binding of ^{125}I-IGF-1 in the MCF 7/5–23 cell line (Figure 2A), suggesting that the increase in binding was mediated via the ER. Specific ^{125}I-IGF-1 binding measured in the presence of either αIR-3 or insulin, both of which do not bind to IGFBP, demonstrated that after treatment with E_2 the increase in ^{125}I-IGF-1 in MCF 7/5–23 cells was predominantly to the IGF-1R (Figure 2B). Again, this effect of E_2 on ^{125}I-IGF-1 binding to the IGF-1R was not observed with the MCF 7/5–21 cell line (data not shown).

Northern blot analysis detects the up-regulation of IGF-1R gene expression by E_2 in tamoxifen-resistant breast cancer cells

To examine whether the effects of E_2 on ^{125}I-IGF-1 binding correlated with an effect on the expression of IGF-1R mRNA, we carried out Northern blot analyses of total RNA isolated from MCF 7/5–21 and 7/5–23 cells as described in the Materials and methods. The hybridization of the IGF-1R probe to both the 11 and 7 kilobase mRNA transcripts described in human tissues (Ullrich et al, 1986) is evident in MCF 7/5–23 cells as illustrated in Figure 3. The rat IGF-1R cDNA probe was used to detect hIGF-IR mRNA in these cell lines as it shares 94% sequence homology to its human counterpart (Werner et al, 1989). In the tamoxifen-resistant MCF 7/5–23 cells, 4–24 h of exposure to 1 nM E_2 in SF medium resulted in a rapid induction of IGF-1R mRNA expression (Figure 3). This effect was still evident when expression of IGF-1R

mRNA was measured after 120 h of E_2 exposure (Figure 3) but was not as marked as in the shorter time course. No effect of E_2 in the tamoxifen-sensitive MCF 7/5–21 cells was observed at any time period (data not shown). When the MCF 7/5–23 cells were incubated with E_2 and tamoxifen simultaneously for 120 h, the induction of the IGF-1R mRNA was moderately inhibited (Figure 3), a result which correlated with data from the IGF-1 binding assays over the same time-course. The effect of tamoxifen alone on IGF-1R mRNA levels in MCF 7/5–23 cells was not significantly different from vehicle controls overall (three experiments).

RT-PCR analysis detects IGF-2 mRNA induced by E_2 in both breast cancer cell lines but no IGF-1 gene expression

Northern data analysis revealed no IGF-1 or IGF-2 mRNA in either cell line grown in SF conditions (data not shown). However, the highly sensitive RT-PCR technique was able to detect IGF-2 expression in both the MCF 7/5–21 cells and the MCF 7/5–23 cells when both cell lines were incubated with vehicle (0.1% ethanol) in SF medium (Figure 4). Relative to the corresponding H3.3 levels for each sample, the basal expression of IGF-2 was increased by 5 days of E_2 exposure in both the MCF 7/5–21 cells (161 ± 4%) and the MCF 7/5–23 cells (240 ± 42%). Co-incubation of both cell lines with E_2 and tamoxifen reduced the expression of IGF-2 to near basal levels. Under no condition did we detect the expression of IGF-1 mRNA in either cell line using RT-PCR (data not shown).

αIR-3 inhibits the growth of only tamoxifen-resistant breast cancer cells

Experiments measuring the effect of the IGF-1R mAb, αIR-3, on the rate of cell proliferation in stock medium demonstrated that whereas the antibody had no significant effect on MCF 7/5–21 cell
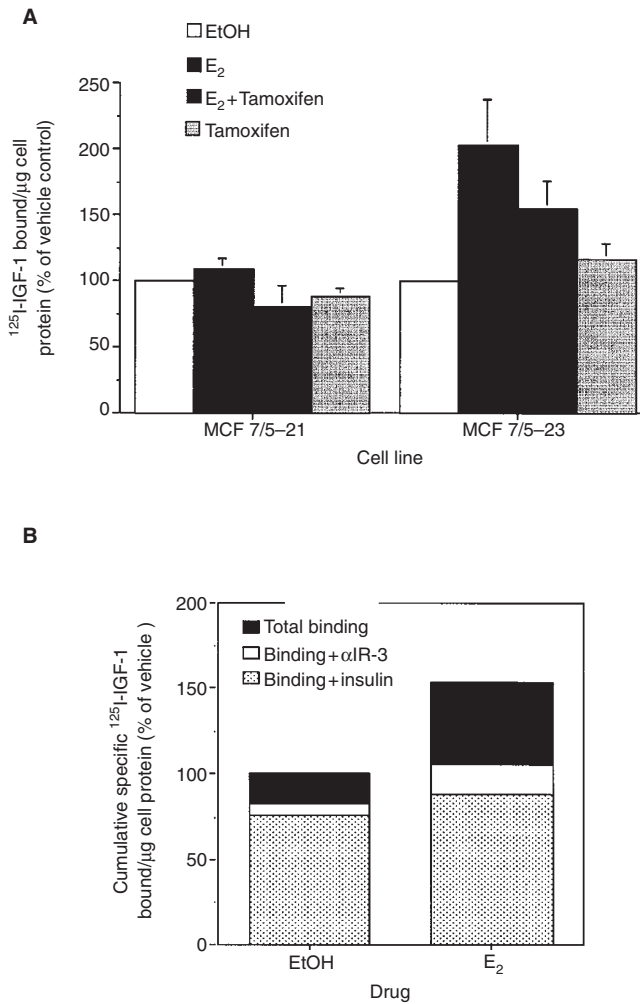
A

B

Figure 2 The effect of E_2 and tamoxifen on IGF-1 binding to breast cancer cells in serum-free conditions. After the cells were withdrawn from oestrogens as described in Materials and methods, binding of ^{125}I-IGF-1 was measured after 5 days incubation in SF medium with the following additions: vehicle (0.1% ethanol), E_2 (10^{-9} M), E_2 (10^{-9} M) + tamoxifen (10^{-6} M) and tamoxifen (10^{-6} M). Near-confluent monolayers of MCF 7/5–21 and 7/5–23 cells were incubated for 16 h at 4°C with 10–20 pM of ^{125}I-IGF-1 ± 100 nM IGF-1 and 50 μM insulin to determine specific, total binding (**A**) or plus αIR-3 (10 μg ml^{-1}) or insulin (50 μM) to determine specific binding to the IGF-1R or IGFBP components in the MCF 7/5–23 cell line (**B**). The cells were washed, lysed and radioactivity counted. Results are expressed as a % of the vehicle control values and are normalized for cell protein content. Each bar represents the mean ± SEM of six separate experiments with a significant ($P < 0.05$) difference in IGF-1 binding for the group E_2 vs EtOH in MCF 7/5–23 cells only
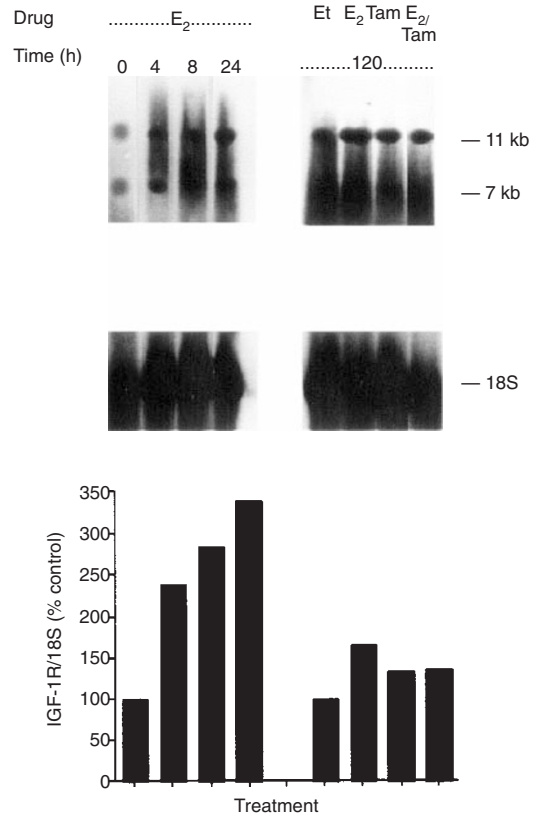

Figure 3 Northern blot analyses of IGF-1R mRNA expression in MCF 7/5–23 cells. Total RNA was extracted from cells withdrawn from oestrogens and treated in SF medium for 0–120 h with the following additions as indicated: vehicle (0.1% ethanol), E_2 (10^{-9} M), tamoxifen (10^{-6} M) and E_2 (10^{-9} M) + tamoxifen (10^{-6} M). At least 30 μg of total RNA was size-fractionated by gel electrophoresis, transferred to a nylon filter, hybridized with the α- ^{32}P-dCTP-rIGF-1R cDNA probe, autoradiographed for up to 10 days and quantified by image analysis. Results from the representative experiment are expressed as a % of the vehicle control and have been corrected for loading by normalizing for 18S ribosomal RNA levels. Similar results were observed in repeated experiments

growth (Figure 5), significant growth inhibitory effects were demonstrated in the MCF 7/5–23 cells. The MTT assay was employed in these experiments as a measurement of the antibody's effect on actively dividing cells as opposed to counting the number of viable cells.

DISCUSSION

We have previously demonstrated that compared to its sister subclone MCF 7/5–21, the relative tamoxifen resistance of the MCF 7/5–23 cells was not explained by the loss of expression of the ER or by a lower affinity of the ER for tamoxifen (Parisot et al, 1995). Thus we reasoned that the tamoxifen-resistant phenotype may have been due to the E_2-independent, autonomous growth of

MCF 7/5–23 cells. In order to test this hypothesis and examine the role of mitogens such as E_2 and IGF-1, we measured the growth of cells in defined, serum-free conditions to avoid potential contamination by residual E_2 or other growth factors present in charcoal-stripped FBS. In growth assays completed over a period of 7 days, we were able to demonstrate a proliferative response to E_2 (in the absence of insulin) in both the MCF 7/5–21 and MCF 7/5–23 subclones. In this study, IGF-1 alone was not mitogenic in the MCF 7/5–21 cells but was significantly mitogenic in the MCF 7/5–23 cells (Table 1).

With the exception of earlier studies by Huff et al (1986) and Karey and Sirbasku (1988), more recent data have shown that IGF-1 alone has only a slight effect on MCF 7 cell proliferation. The larger effects of IGF-1 in these earlier studies were most likely due to the low levels of residual E_2 in the culture medium or the presence of the phenol-red dye, a weak E_2 (Berthois et al, 1986). Hence, studies which used a more severe E_2-withdrawal regimen lasting almost a week, as well as phenol-red free medium, observed very small effects of IGF-1 on MCF 7 cell proliferation (Stewart et al, 1990, 1992; Thorsen et al, 1992; Wiseman et al, 1993). However, all of these studies were able to demonstrate a marked synergistic effect of E_2 and IGF-1 on MCF 7 cell proliferation despite the
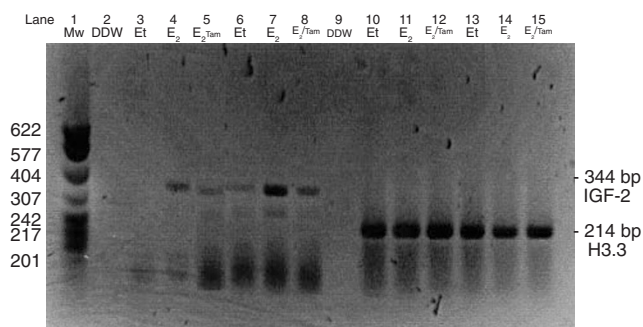
Figure 4 RT-PCR analysis of IGF-II and H3.3 mRNA expression. Poly (A)+ RNA was reverse-transcribed and one-tenth of the reaction volume was amplified with specific primers for IGF-2 and histone 3.3 for 35 cycles. Following PCR, the amplified products were electrophoresed in 1.5% agarose gels stained with ethidium bromide (0.5 mg ml) and the fluorescence quantified with image analysis software. Legend: pBR322/*Msp*1 Mw ladder (lane 1); DDW (lane 2, 9), MCF 7/5–21 cells and MCF 7/5–23 cells incubated with vehicle (0.1% ethanol), E_2 (10^{-9} M) and E_2 (10^{-9} M) + tamoxifen (10^{-6} M), respectively, with amplification for IGF-2 (5–21: lanes 3–5; 5–23: lanes 6–8) and histone 3.3 (5–21: lanes 10–12; 5–23: lanes 13–15). Data from this representative experiment was similar to those observed in repeated experiments.

Figure 5 Inhibition of cell growth by the IGF-1R monoclonal antibody (αIR-3). MCF 7/5–21 and 7/5–23 cells were plated and grown for 48 h in RPMI-1640 with 10% FBS before the addition of αIR-3 (1 μg ml^{-1}). Five days later, the rate of cell proliferation was determined by spectrophotometric measurement of MTT dye reduction. Results are expressed as a % of the absorbance measured in the presence of vehicle only (1 μg ml^{-1} mouse IgG$_1$) with each bar representing the mean $\pm$ SEM of two separate experiments with a significant ($P < 0.05$) difference for MCF 7/5–23 cells only.

insignificant effect of IGF-1 alone, as was observed in this study with the MCF 7/5–21 cells.

This synergy between E_2 and IGF-1 on ER+ breast cancer cell proliferation has been previously attributed to the up-regulation of the IGF-1R by E_2 (Stewart et al, 1990; Westley and May, 1994; Huynh et al, 1996; Clarke et al, 1997). Similarly, our results demonstrated that the addition of E_2 doubled ^{125}I-IGF-1 binding in the tamoxifen-resistant MCF 7/5–23 cells over a 5-day period. That the E_2-induced increase in ^{125}I-IGF-1 binding in MCF 7/5–23 cells was due to the induction of IGF-1R mRNA by E_2 was confirmed using Northern blot analyses. It was found that E_2 exposure for 24 h increased the expression of IGF-1R mRNA about threefold in MCF 7/5–23 cells and was still relatively elevated 4 days later, data which correlated with the results of the binding assay. Yet, peculiarly, we were unable to demonstrate this effect in the tamoxifen-sensitive MCF 7/5–21 cells.

The role of the IGFBP cannot be discounted as it may mask changes in the level of the IGF-1R in the MCF 7/5–21 cells when measured by ^{125}I-IGF-1 binding assays. However, preliminary examination of specific ^{125}I-IGF-1 binding to the IGF-1R by using the αIR-3 antibody or a high concentration of insulin revealed that there was no effect of E_2 in the MCF 7/5–21 cells but ^{125}I-IGF-1 binding to the IGF-1R was still increased in the MCF 7/5–23 cells. Hence, the induction of ^{125}I-IGF-1 binding was most probably indicative of an increment in IGF-1R number and not a measurement of membrane-associated IGFBP activity. Nevertheless, we did observe that up to 80% of specific ^{125}I-IGF-1 binding in these MCF 7 subclones was to the IGFBP as has been previously reported (Kleinman et al, 1995).

The data also demonstrate that IGF-1R signalling is activated during the culture of either of the subclones in serum-containing medium because of the ability of the IGF-1R mAb, αIR-3, to inhibit the growth of the cells. Although the inhibition of MCF 7/5–21 cell growth by αIR-3 was not significant, the mean level of cell growth inhibition was 20%, a range comparable to that observed with the MCF 7 cells at a concentration of 1 μg ml^{-1} antibody (Rohlik et al, 1987; Arteaga and Osborne, 1989). In contrast, αIR-3 (1 μg ml^{-1}) significantly inhibited the growth of the MCF 7/5–23 cells by 50%, implicating a higher level of IGF-1R activity in this cell line.
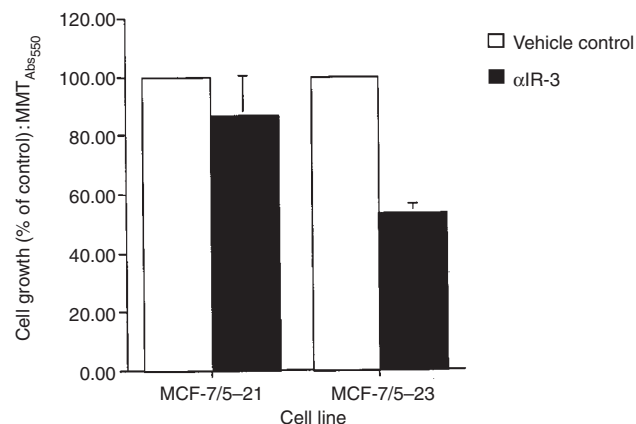
Although others have reported over-expression of the IGF-1R in breast cancer (Pekonen et al, 1988; Papa et al, 1993) there are only limited data regarding the association of this finding with the development of tamoxifen resistance in ER+ breast cancer (Wiseman et al, 1993). Decupis et al (1995) observed that, although E_2 increased the number of IGF-1R in tamoxifen-resistant MCF 7/LCC2 cells, it did not do likewise in tamoxifen-resistant MCF 7/LY-2 cells. Similarly, van den Berg et al (1996) observed that E_2 failed to up-regulate the IGF-1R in the tamoxifen-resistant ZR-75-9al cells, which expressed a lower level of the IGF-1R (McCotter et al, 1996). This difference between the tamoxifen-resistant cell lines is presumably related to the level of expression of the ER and the degree of E_2 responsiveness.

Wiseman et al (1993) suggested that the development of tamoxifen resistance occurred via the agonistic (oestrogenic) properties of tamoxifen increasing the expression of the IGF-1R. They reported an increase in ^{125}I-IGF-1 binding by tamoxifen in a tamoxifen-resistant subclone of MCF 7 cells. In contrast, I detected no agonistic effects of tamoxifen in the MCF 7/5–23 cells. However, our data indicated that tamoxifen was unable to completely inhibit E_2-induced ^{125}I-IGF-1 binding to basal levels. When tamoxifen was co-incubated with E_2, the resultant ^{125}I-IGF-1 binding was partially inhibited compared with E_2 alone (Figure 2A) and tamoxifen partially inhibited E_2-induced IGF-1R mRNA expression (Figure 3), suggesting that tamoxifen was predominantly acting as an E_2 antagonist in these cells and under these conditions but was not fully effective.

A possible explanation for the difference in these observations may be the methods used to generate these tamoxifen-resistant cell lines. Although the results of Wiseman et al (1993) were obtained with subclones selected in the continued presence of tamoxifen, the tamoxifen-resistant MCF 7/5–23 cell line reported herein represented a spontaneously generated subclone obtained by maintaining the genetically unstable MCF 7 cells in continuous exponential growth (Reddel et al, 1988). No other selective pressure was used to achieve tamoxifen resistance (defined relative to its sister subclone MCF 7/5–21, generated concurrently).

Many investigators have shown an increase in ^{125}I-IGF-1 binding or IGF-1R mRNA after E_2 treatment in the MCF 7 cell

line (Freiss et al, 1990; Stewart et al, 1990; Decupis et al, 1995; Huynh et al, 1996; Clarke et al, 1997). It is unclear to us why E_2 failed to increase ^{125}I-IGF-1 binding or IGF-1R mRNA levels in the MCF 7/5–21 cells, given that their rate of proliferation was also increased synergistically by co-incubation with E_2 and IGF-1. Another possible explanation could be that the synergy between E_2 and IGF-1 is also dependent on other mechanisms besides the up-regulation of the IGF-1R by E_2. Recently, it has been proposed that the synergy between E_2 and IGF-1 may be mediated by increased activation of the ER through IGF-1R cross-talk. Stimulation of the IGF-1R has been implicated in the increase of ER-phosphorylation through the Ras-MAPK cascade of the IGF-1R signalling pathway (Kato et al, 1995) resulting in enhanced ER-mediated cell proliferation.

The detection of IGF-1R mRNA expression in the MCF 7/5–23 cells and their sensitivity to IGF-1 lead us to question whether the IGF-mediated proliferation of these cells was via an autocrine mechanism. However, we could detect no IGF-1 mRNA expression using firstly Northern blot analysis or the more sensitive RT-PCR method. In fact, IGF-1 mRNA has not been detected using RT-PCR in a large number of breast cancer cell lines (Quinn et al, 1996), although IGF-2 mRNA has been detected both in T47D cells and late passage MCF 7 cells (Yee et al, 1988). Using RT-PCR, we demonstrated that both of our subclones expressed IGF-2 mRNA and we were able to identify a significant, E_2-induced increase in IGF-2 expression primarily in the MCF 7/5–23 cells (Figure 4). Thus, IGF-2, a ligand that has a moderately high affinity for the IGF-1R, may act as an autocrine regulator of cell growth in these cell lines but we have not as yet demonstrated the presence of immunoreactive IGF-2. This may be especially important for the MCF 7/5–23 cells, which we have shown are responsive to IGF-1 and express IGF-1R that is up-regulated by physiological concentrations of E_2. Interestingly, immunoreactive IGF-2 activity has been detected in MCF 7 (Lee et al, 1994*a*) and T47D cells (Osborne et al, 1989) and in a tamoxifen-resistant MCF 7 subclone (Lee et al, 1994*b*) developed by Toi et al (1993).

Finally, in MCF 7/5–23 cells the up-regulation of IGF-1R expression by E_2 only partially inhibited by tamoxifen combined with the sensitivity of these cells to IGF-1, may be the explanation for the relative tamoxifen-resistance of MCF 7/5–23 cells compared to MCF 7/5–21 cells which are unable to bypass growth inhibition by tamoxifen through the IGF-1R pathway.

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