Increased expression of both insulin receptor substrates 1 and 2 confers increased sensitivity to IGF-1 stimulated cell migration

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Abstract

Insulin-like growth factors (IGFs) are thought to promote tumour progression and metastasis in part by stimulating cell migration. Insulin receptor substrate-1 (IRS-1) and IRS-2 are multisite docking proteins positioned immediately downstream from the type I IGF and insulin receptors. IRS-2 but not IRS-1 has been reported to be involved in the migratory response of breast cancer cells to IGFs. The purpose of this investigation was to determine if IRS-1 is involved in, and to assess the contributions of IRS-1 and IRS-2 to, the migratory response of breast cancer cells to IGFs. The expression of IRS-1 and IRS-2 varied considerably between ten breast cancer cell lines. Oestrogen increases expression of the type I IGF receptor, IRS-1 and IRS-2 in MCF-7 and ZR-75 cells. Oestrogens may control the sensitivity of breast cancer cells to IGFs by regulating the expression of components of the IGF signal transduction pathway. The migratory response to a range of IGF-1 concentrations was measured in MCF-7 and MDA-MB-231 breast cancer cells in which IRS-1 and IRS-2 levels were modulated using a doxycycline-inducible expression system. Induction of both IRS-1 and IRS-2 expression increased the sensitivity of the migratory response to IGF-1 but did not increase the magnitude of the response stimulated at higher concentrations of IGF-1. Knockdown of IRS-1, IRS-2 and the type I IGF receptor in MCF-7 and MDA-MB-231 cells decreased sensitivity to IGF-1. We conclude that both IRS-1 and IRS-2 control the migratory response of breast cancer cells to IGF-1 and may, therefore, be key molecules in determining breast cancer spread.

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Introduction

The insulin-like growth factor (IGF) system comprises two ligands (IGF-1 and IGF-2) as well as the closely related hormone insulin, six binding proteins (IGFBP1–6), three receptors (type I and type II IGF and the insulin receptors) and immediate downstream signalling proteins. The insulin receptor substrate (IRS) proteins act as multisite docking molecules for downstream effectors that have src homology (SH2) domains such as the p85 regulatory subunit of phosphatidylinositol 3 kinase (PI3-kinase) and Shc. Thus, activation of IRS proteins is an early step in various complex signalling cascades that lead to the biological effects of IGFs (Sachdev & Yee 2001). The IGFs are, as their name suggests, related to insulin.

The structures of the type I IGF and insulin receptors are similar and both are heterotetrameric trans-membrane ligand-activated protein tyrosine kinases. The insulin and type I IGF receptors are sufficiently closely related to form hybrid receptors whose signalling characteristics differ from those of the parent receptors (Pandini *et al.* 1999). The type I IGF receptor binds IGF-1 and IGF-2 with high affinity and insulin with lower affinity.

The IGFs were discovered as a result of their key roles in juvenile growth and development. The major source of circulating IGFs is the liver and their synthesis is controlled by GH. The IGFs are also synthesised at extrahepatic sites where they act as paracrine and autocrine growth factors.

IGFs have been implicated in the aetiology and progression of a variety of cancers (Pollak 2000). Elevated circulating levels of IGF-1 together with decreased concentrations of IGFBP3, the protein to which it is bound in the circulation, are a significant risk factor for breast (Hankinson *et al.* 1998, Davies *et al.* 2006, Schernhammer *et al.* 2005), colon (Davies *et al.* 2006) and prostate cancer (Chan *et al.* 1998). Over expression of components of the IGF system such as the type I IGF receptor and IRS-1 and IRS-2 are characteristic of some cancers and can result in cell transformation in experimental systems (D'Ambrosio *et al.* 1995, Guvakova & Surmacz 1997, Chang *et al.* 2002). In addition, there is evidence that elevated IRS-1 expression is associated with a poor prognosis in breast cancer (Rocha *et al.* 1997).

The normal biological properties of IGFs are thought to be subverted by cancer cells to facilitate tumour progression. IGFs are mitogenic for a large number of cell types including breast cancer cells (Stewart *et al.* 1990, Daws *et al.* 1996), they inhibit apoptosis induced by a variety of agents including staurosporine and ceramide (Peruzzi *et al.* 1999, Kooijman 2006) and they increase cell migration and invasion (Doerr & Jones 1996, Bartucci *et al.* 2001). All of these properties are required for the growth and metastasis of tumour cells (Baserga 2004).

On the basis of a large amount of biological data, the IGF system is emerging as a credible therapeutic target in cancer (Baserga 2004, Yee 2006). A number of strategies are in development of which the most important are inhibition of the binding of IGF to the receptor and down regulation of the type I IGF receptor by anti-receptor antibodies (Li *et al.* 2000, Hailey *et al.* 2002, Maloney *et al.* 2003, Sachdev *et al.* 2003, Wang *et al.* 2005) and inhibition of the receptor tyrosine kinase (Stromberg *et al.* 2006, Tazzari *et al.* 2007).

In breast cancer, lifetime exposure to oestrogens is a principal risk factor (Eliassen *et al.* 2006) and the oestrogen receptor (ER) is an important therapeutic target as exemplified by the development and widespread therapeutic use of oestrogen antagonists (Wickerham 2002) and aromatase inhibitors (Altundag & Ibrahim 2006). The mechanisms by which oestrogens control breast cancer progression are not completely understood but the genomic effects of oestrogens whereby they control the expression of a limited repertoire of genes are of major importance (Westley & May 2006). We (Stewart *et al.* 1990, Daws *et al.* 1996) and others (Lee *et al.* 1999, Mauro *et al.* 2001, Bernard *et al.* 2006) have shown that oestrogens increase the responsiveness of breast cancer cells to IGFs and that they regulate the expression of

key components of the IGF signal transduction pathway including IGF-2 (Westley *et al.* 1998) the type I IGF receptor (Stewart *et al.* 1990) and IRS-1 (Molloy *et al.* 2000). This has led to the concept that oestrogens may act, at least in part, through increasing the responsiveness of breast cancer cells to IGFs.

The stimulation of breast cancer cell migration by IGFs may be one mechanism by which oestrogens promote tumour progression. The mitogenic effects of IGFs have, however, been somewhat controversial. Bae *et al.* (1993) concluded that the MCF-7 cell line which expresses relatively high levels of the type-1 IGF receptor has a non-invasive phenotype while Pennisi *et al.* (2002) implicated the type-1 IGF receptor in cell migration but found that receptor expression is inversely related to cell migration. Jackson *et al.* (2001) showed that IGF-1 stimulated the migration of a metastatic variant of MDA-MB-231 cells but not the parental cells. By contrast, Doerr & Jones (1996), Bartucci *et al.* (2001) and Byron *et al.* (2006) showed that IGFs do stimulate the migration of breast cancer cell lines. One aim of this study therefore was to determine whether IGFs stimulate breast cancer cell migration and we conclude that there is a concentration dependent increase in cell migration in both MCF-7 and MDA-MB-231 cell lines.

Recent studies have reported distinct roles for IRS-1 and IRS-2 in breast cancer and suggested that IRS-1 regulates cell proliferation whereas IRS-2 regulates cell migration (Jackson *et al.* 2001, Byron *et al.* 2006, Gibson *et al.* 2007). In the present study, we examine the possible role of IRS proteins in mediating the effects of oestrogen on breast cancer cell invasion by increasing specifically IRS-1 or IRS-2 expression in breast cancer cells using a tetracycline-inducible expression system. Notably, we show that IRS-1 as well as IRS-2 can increase cell migration. This focuses attention on both IRS-1 and IRS-2 as a convergence point of oestrogen and IGF signalling and as potential therapeutic targets for the treatment of hormone responsive and non-responsive breast cancer.

Materials and methods

Cell culture

MCF-7 cells, their derivatives and all other breast cell lines, ZR-75, T-47D, EFF-3, EFM-19, BT 20, Hs 578T, SK-BR-3 and HBL-100, apart from MDA-MB-231 were cultured routinely in DMEM containing 10% FCS and 1 µg/ml (0.17 µM) insulin. MDA-MB-231 cells and derivatives were cultured in DMEM containing 5% FCS and 1 µg/ml (0.17 µM) insulin.

Preparation of stable pTet-on cell lines

MCF-7 cells, stably transfected with the pTet-on plasmid, were purchased from BD Clontech. MDA-MB-231 cells stably transfected with the pTet-on plasmid were created using liposome-mediated transfection (Clontectin, BD Clontech) and selection in 400 µg/ml G418. Clones were transiently transfected with the pTRE2-luc plasmid (BD Clontech) using liposome-mediated transfection and the clone with the highest induction of luciferase by doxycycline and the lowest background was used in subsequent experiments.

Cloning of the IRS-1 and IRS-2 cDNA into the pTRE2 response plasmid

The human IRS-1 (Sun *et al.* 1991) and IRS-2 (Vassen *et al.* 1999) mRNA sequences were excised from the recombinant plasmids, purified on a low melting agarose gel and then ligated to pTRE2 plasmid (BD Clontech). Recombinant plasmids were sequenced to confirm the correct sequence and orientation of the cDNA.

Creation of Tet-on cells stably transfected with recombinant pTRE2 plasmids

MCF-7 Tet-on and MDA-MB-231 Tet-on cells were co-transfected with the recombinant pTRE2 plasmids and pTKhyg which confers resistance to hygromycin. Stably transfected cell lines were selected by culture in hygromycin B. The expression of IRS-1 and IRS-2 was measured in the transfected MCF-7 and MDA-MB-231 cells following culture in the presence of doxycycline for 48 h in three separate experiments. IRS-1 expression was increased threefold (3 ± 0.5) and IRS-2 expression fivefold (5 ± 0.4) in MCF-7 cells, whereas IRS-1 was increased fourfold (4 ± 0.6) and IRS-2 expression sevenfold (7 ± 0.7) in MDA-MB-231 cells. The expression of the type I IGF receptor and insulin receptor in MCF-7 and MDA-MB-231 cells transfected with pTRE2 plasmid, pTRE2 IRS-1 plasmid or pTRE2 IRS-2 plasmid was not affected by treatment with 50 ng/ml (6.5 nM) IGF-1, 50 ng/ml (8.5 nM) insulin or 1 µg/ml (0.17 mM) insulin (data not shown).

Measurement of type I IGF receptor, IRS-1 and IRS-2 proteins by western transfer

Lysates were prepared either from cells growing in normal culture medium or from cells that had been stimulated with combinations of 17β-oestradiol and IGF-1. For the latter, cells were grown in T25 flasks until ~60% confluent, washed twice with PBS and

cultured for 6 days in phenol red-free DMEM supplemented with 10% charcoal-treated newborn calf serum. The medium was changed every 24 h. To study the effects of oestrogen, cells were cultured for an additional 2 days in the above medium or medium supplemented with the combinations of 10 nM oestrogen, 50 ng/ml (6.5 nM) IGF-1 and 1 µg/ml (0.17 µM) insulin for 48 h. After 48 h, cells were washed with ice-cold PBS, and lysed using RIPA buffer containing a cocktail of protease and phosphatase inhibitors. The lysate was centrifuged at 12 000 *g* at 4 °C for 10 min, the supernatant removed and stored frozen. Protein concentrations were measured using the bicinchoninic acid assay (Pierce Protein Research Products, Thermo Fisher Scientific Inc., Rockford, IL, USA) with BSA as the standard. Expression was evaluated in a minimum of three experiments that were analysed at least twice and in each case a representative result is shown in the figures for illustrative purposes.

Cell lysate proteins were separated on a 9 or 10% acrylamide separating gel or on a gradient polyacrylamide gel with a 3% acrylamide stacking gel and transferred to nitrocellulose. The nitrocellulose filters were blocked using tris-buffered saline containing 5% milk and 0.1% Tween 20 (TBST-milk) for 1 h at room temperature and then incubated overnight with anti IRS-1 (1:1000–1:5000), anti IRS-2 (1:500–1:1000), anti type I IGF receptor (1:1000–1:4000) or anti GAPDH (1:6000–1:20 000) in TBST-milk overnight at 4 °C. The antibodies were from Upstate, Millipore, Billerica, MA, USA (IRS-1, 06–248; IRS-2, 06–506), Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (IRS-1 sc-7200; IRS-2, sc-1555-R; GAPDH, sc-25778) and Cell Signaling Technology Inc., Boston, MA, USA (type I IGF receptor, 3027). The filters were washed four times in TBST and incubated in HRP-conjugated goat-anti-rabbit antibody (1:2000) and the proteins detected using enhanced chemiluminescence (Western Dura Super Signal extended duration substrate, Pierce). Protein expression was quantified by densitometric analysis of the resultant X-ray films and the optical density analysed with Labworks 4 software (Ultraviolet Products, Cambridge, UK). Data were normalised by reference to expression of GAPDH.

Measurement of cell migration

Insulin binds to and stimulates auto-phosphorylation of the type I IGF receptor. The medium used for the routine culture of breast cancer cell lines contains insulin at a concentration high enough (1 µg/ml, 0.17 µM) to stimulate the type I IGF receptor. We were therefore concerned that the migration of cells

may be affected by the culture conditions and in particular that it could be affected by the removal of ligands for the type I IGF receptor prior to the migration assay. The response to IGF-1 was therefore measured in cells grown in the absence or presence of IGF-1 for the 48 h prior to the migration assay (data not shown). There were no statistically significant differences between the responses of cells cultured in the presence or absence of IGF-1 for 48 h prior to the migration assay and cells were cultured in the absence of IGF-1 in all subsequent experiments.

Cells were grown in T25 flasks until ~30% confluent, washed twice with PBS and cultured for 48 h in phenol red-free DMEM supplemented with 10% charcoal-treated newborn calf serum in the presence or absence of doxycycline (2 µg/ml). Cells were trypsinised after 48 h and 1.5×10^4 cells added to the upper chamber of a micro chemotaxis chamber in 50 µl of phenol red-free DMEM containing 0.01% BSA. The upper and lower chambers were separated by an 8 µm pore membrane that had been coated with collagen IV for 45 min at room temperature. The bottom chamber contained phenol red-free DMEM supplemented with 0.01% BSA containing varying concentrations of IGF-1 (0–50 ng/ml; 0–6.5 nM). The response to each concentration of IGF-1 was tested in triplicate assays in each experiment. The chamber was incubated for 5 h (MDA-MB-231 and derivatives) or 6 h (MCF-7 and derivatives) at 37 °C in a cell culture incubator. The chamber was then dismantled, cells scraped from the upper surface and the migrated cells attached to the lower surface, by fixation in methanol for 5 min. The cells were stained with haematoxylin for 5 min, the filter washed under running tap water and in Scott's tap water for 5 min and then mounted on glass slides with the migrated cells on the uppermost surface. Cell migration was quantified by counting the migrated cells in five fields at 400-fold magnification in each of the triplicate assays (Prest *et al.* 2002).

Knockdown of type I IGF receptor, IRS-1 and IRS-2

MCF-7 and MDA-MB-231 cells were transfected with empty vector, or siRNA plasmids: pKD-IGF-IR-v2; pKD-IRS1-v3 or pKD-IRS2-v4 (Upstate) in the presence of FuGENE HD transfection reagent (Roche) at different ratios as recommended by the manufacturer. After 18–24 h, cells were lysed or the medium was replaced with routine culture medium. Thereafter, the medium was changed daily and cells were lysed at daily intervals. Aliquots of protein lysate were analysed by western transfer for expression of the

type I IGF receptor, IRS-1 or IRS-2. Optimal knock-down to 10–20% of the expression of the proteins in the control transfected cells was obtained after 2–3 days in MCF-7 cells and after 2–3 days in MDA-MB-231 cells.

To test the effects of the knockdown on migration, cells were grown to 20–30% confluence in T25 flasks, washed with PBS and transfected with the siRNA plasmids at a 3:1 ratio of FuGENE HD to DNA. After 18–24 h, the cells were washed twice with PBS and the medium was replaced with phenol red-free DMEM supplemented with 10% charcoal-treated newborn calf serum and the cells were cultured for 24 h. The migration response of MDA-MB-231 cells was tested at this stage but MCF-7 cells were washed twice with PBS and the medium was replaced with phenol red-free DMEM supplemented with 10% charcoal-treated newborn calf serum and cultured for a further 24 h at which time the migration response was measured essentially as described above. Concentrations of IGF-1 between 0 and 100 ng/ml (0–13 nM) were tested, the chambers were incubated for 4–4.5 h and the non-migrated cells were allowed to remain on the filters.

Statistical analysis

Differences between groups were tested by ANOVA, paired or unpaired *t*-test and Mann–Whitney test.

Results

Expression of IRS-1 and IRS-2 in human breast cancer cell lines

IRS-1 and IRS-2 expression was measured by western transfer analysis in a panel of 10 human breast cancer cell lines comprising five oestrogen responsive (MCF-7, T-47D, ZR-75-1, EFM-19, EFF-3) and five oestrogen non-responsive lines (HBL-100, Hs 578T, MDA-MB-231, SK-BR-3 and BT 20; Fig. 1A and B) that had been cultured in normal growth medium. The levels of expression of both proteins varied by approximately two orders of magnitude in the ten cell lines. Expression of IRS-1 was highest in the oestrogen non-responsive Hs 578T cell line whereas expression of IRS-2 was highest in the oestrogen non-responsive MDA-MB-231 cell line.

Effect of IGF-1 on cell migration

The effects of different concentrations of IGF-1 were tested on cell migration in both MCF-7 and MDA-MB-231 cells that had been stably transfected with the pTet-on plasmid alone (Fig. 2). The effect of IGF-1 on cell migration was tested on cells that had

Figure 1 Expression of IRS-1 and IRS-2 in human breast cancer cells. HBL-100 (HBL); Hs578T (Hs); MCF-7 (MCF); T-47D (T47); ZR-75 (ZR); EFM-19 (EFM); Sk-Br-3 (SkBr); EFF-3 (EFF) and BT 20 (BT20) cells were grown in DMEM containing 10% FCS and 1 μ g/ml insulin. MDA-MB-231 (MDA) cells were grown in DMEM containing 5% FCS and 1 μ g/ml insulin. The cells were lysed and protein extracts prepared as described in the Materials and methods. The amounts of IRS-1 (A) and IRS-2 (B) were measured in these oestrogen responsive and non-responsive breast cell lines by western transfer analysis as described in the Materials and methods. Aliquots of 10 μ g protein were electrophoresed on 9% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with specific IRS-1 antibody (Upstate 06-248) or specific IRS-2 antibody (Upstate 06-506) overnight at 4 °C, HRP-conjugated goat-anti-rabbit antibody and the proteins detected using enhanced chemiluminescence (Western Dura Super Signal extended duration substrate, Pierce). The amount of each protein was determined by densitometric scanning of X-ray films followed by analysis with Labworks 4 software as described in the Materials and methods. The amount of each protein was expressed as the percentage of the amount of protein in the cell line expressing the most protein and is represented in the histogram. The filled and unfilled columns indicate the expression in oestrogen-responsive and oestrogen non-responsive cell lines respectively.

Figure 2 Effect of IGF-1 on cell migration of MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were cultured for 48 h in phenol red-free DMEM supplemented with 10% charcoal-treated newborn calf serum. Cells were trypsinised after 48 h, washed and 1.5×10^4 cells added to the upper chamber of a micro chemotaxis chamber in 50 μ l phenol red-free DMEM containing 0.01% BSA. The bottom chambers contained different concentrations of IGF-1 in 30 μ l phenol red-free DMEM containing 0.01% BSA. Squares show the migration of MDA-MB-231 cells and circles the migration of MCF-7 cells stimulated by different concentrations of IGF-1 and analysed as described in the Materials and methods. The bars show the S.E.M. of measurements from four separate experiments. In both cell lines, IGF-1 stimulated migration at and above 10 ng/ml (1.3 nM). Asterisks show migration that is statistically significantly higher than in control cells for each cell line using ANOVA (10 ng/ml (1.3 nM) and above, $P < 0.05$). The MCF-7 Tet-on cells show significantly more migration than MDA-MB-231 Tet-on cells at 10 ng/ml (1.3 nM) IGF-1 (t -test, $P = 0.01$).

been cultured in the absence of insulin for two days. The migration of the MCF-7 cells was increased approximately tenfold by IGF-1. The increase was concentration dependent with a half maximal increase at 8 ng/ml (1.04 nM). The effect of IGF-1 on the migration of the MDA-MB-231 cells was tested using the same culture conditions. IGF-1 increased MDA-MB-231 cell migration approximately tenfold with half maximal stimulation at a concentration of 12 ng/ml (1.62 nM). While the magnitude of the effect of IGF-1 on migration in the two cell lines was similar, the stimulation at 10 ng/ml (1.3 nM) IGF-1 was significantly ($P = 0.048$) greater in MCF-7 Tet-on cells than in MDA-MB-231 Tet-on cells.

Effects of oestrogen, IGF-1 and insulin on the induction of type I IGF receptor, IRS-1 and IRS-2 in oestrogen-responsive breast cancer cells

IGF-1 is a high-affinity ligand whereas insulin is a low-affinity ligand for the type I IGF receptor. The effects of oestrogen in the absence and presence of

IGF-1 and insulin on the expression of the type I IGF receptor, IRS-1 and IRS-2 were measured in two oestrogen-responsive breast cancer cell lines to assess the induction by oestrogen and the effects of insulin and IGF-1 on the oestrogen induction (Fig. 3). The effects of oestrogen were tested also in the MDA-MB-231 oestrogen non-responsive cell line. The type I IGF receptor antibody detected two bands; a minor band of ~165 kDa which corresponds most probably to the proprotein of the type I IGF receptor and a major band of 95 kDa corresponding to the processed β -subunit. Oestrogen induced expression of the type I IGF receptor in the absence and presence of IGF-1 and insulin and there was no synergistic effect of oestrogen with either type I IGF receptor ligand. The expression of the type I IGF receptor was greater in MCF-7 than in ZR-75 cells but its induction by oestrogen was more marked in ZR-75 cells.

IRS-1 was induced dramatically by oestrogen in both MCF-7 and ZR-75 cells. It was expressed at higher levels in oestrogen-treated MCF-7 cells. In

MCF-7 cells, IRS-1 induction was greater in the presence of IGF-1 and insulin than with oestrogen alone and insulin and IGF-1 increased IRS-1 slightly in the absence of oestrogen. IRS-2 was induced by oestrogen in both MCF-7 cells and ZR-75 cells and induction was greater in ZR-75 cells. IGF-1 and insulin decreased slightly IRS-2 expression in the absence of oestrogen in ZR-75 cells, therefore the induction by oestrogen was greater in their presence in this cell line. Oestrogen had no effect on the expression of the type I IGF receptor, IRS-1 or IRS-2 in MDA-MB-231 cells.

The induction by oestrogen of the type I IGF receptor, IRS-1 and IRS-2 varies with the cell line and culture conditions as shown previously for other oestrogen responsive genes (Clayton *et al.* 1997, Donaghue *et al.* 1999, Westley & May 2006). These experiments show, however, that these key components of the IGF signal transduction pathway are all induced by oestrogen in both oestrogen-responsive breast cancer cell lines.

Figure 3 Effect of oestrogen on the expression of the type I IGF receptor, IRS-1 and IRS-2 in oestrogen-responsive breast cancer cells. MCF-7 and ZR-75 oestrogen-responsive breast cancer cells and MDA-MB-231 oestrogen non-responsive breast cancer cells were withdrawn from hormones present in routine culture medium and treated with oestradiol (E_2 , 10 nM) either alone or in the presence of IGF-1 (50 ng/ml; 6.5 nM) or insulin (1 μ g/ml; 0.17 μ M) for 48 h. The cells were lysed and protein extracts prepared as described in the Materials and methods. The amounts of type I IGF receptor, IRS-1 and IRS-2 were measured by western transfer analysis as described in the Materials and methods. Aliquots of 20 μ g protein were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with specific type I IGF receptor antibody (Cell Signalling, 3027), specific IRS-1 antibody (Upstate 06-248), specific IRS-2 antibody (Santa Cruz, sc-1555-R) or specific GAPDH antibody (Santa Cruz, sc-25778) overnight at 4 °C. The membranes were washed and incubated with HRP-conjugated goat-anti-rabbit antibody and the proteins detected using enhanced chemiluminescence (Western Dura Super Signal extended duration substrate, Pierce). The relative amounts of each protein were determined by densitometric scanning of X-ray films followed by analysis with Labworks 4 software as described in the Materials and methods.

Transfection of breast cancer cell lines with inducible IRS-1 and IRS-2 expression vectors

One oestrogen responsive and one oestrogen non-responsive breast cancer cell line was transfected with an inducible IRS-1 or IRS-2 expression construct to examine the importance of IRS-1 and IRS-2 on stimulation of cell migration by IGF-1. The Tet-on system, in which expression is induced by doxycycline, was used for these experiments.

Recombinant pTRE2 plasmids containing the entire coding region of human IRS-1 (Sun *et al.* 1991) and IRS-2 (Vassen *et al.* 1999) were constructed as described in the Materials and methods. These plasmids were stably transfected into MCF-7 and MDA-MB-231 Tet-on cells. The expression of IRS-1 and IRS-2 was measured in the transfected MCF-7 and MDA-MB-231 cells following culture in the presence of doxycycline for 48 h. IRS-1 expression was increased approximately threefold and IRS-2 expression fivefold in MCF-7 cells, whereas IRS-1 was increased fourfold and IRS-2 expression sevenfold in MDA-MB-231 cells (data not shown).

Effect of increased expression of IRS-1 and IRS-2 on MCF-7 cell migration

The effect of increased expression of IRS-1 and IRS-2 on IGF-stimulated migration was measured in cells that had been cultured in the absence or presence of 2 µg/ml doxycycline for 48 h. Doxycycline had no effect in MCF-7 Tet-on cells (Fig. 4A). Cell migration was increased approximately tenfold and the concentration of IGF-1 required for half-maximal cell migration was 8 ng/ml in the presence and absence of doxycycline.

By contrast, the response of MCF-7 IRS-1 and MCF-7 IRS-2 cells to IGF-1 was altered by treatment with doxycycline. For both cell lines, the number of migrated cells at higher concentrations of IGF-1 was not altered but the concentration of IGF-1 required for

Figure 4 Effect of increased expression of IRS-1 and IRS-2 in MCF-7 cells on IGF-1 stimulated cell migration. MCF-7 cells transfected with pTRE2 (A), pTRE2 IRS-1 (B) and pTRE2 IRS-2 (C) were cultured in the absence and presence of doxycycline and the migration in response to different concentrations of IGF-1 was measured as described in the legend to Fig. 2. Circles show cell migration after culture in the absence of doxycycline, whereas triangles show cell migration after culture in the presence of doxycycline. The bars show the S.E.M. of at least three experiments. Asterisks show the concentrations at which migration is statistically significantly higher in the cells that had been cultured in the presence of doxycycline (*t*-test, $P=0.04$ at 2 ng/ml; 0.26 nM, $P=0.03$ at 5 ng/ml; 0.65 nM for both MCF-7 IRS-1 and IRS-2) than in cells that had been cultured in the absence of doxycycline. The migration was statistically higher in cells stimulated with IGF-1 than in unstimulated cells at IGF-1 concentrations of 20 ng/ml; 2.6 nM and above for MCF-7 cells that had been cultured in the absence of doxycycline, 10 ng/ml; 1.3 nM and above for MCF-7 cells that had been cultured in the presence of doxycycline, 10 ng/ml; 1.3 nM and above for MCF-7 IRS-1 cells that had been cultured in the absence of doxycycline, 2 ng/ml; 0.26 nM and above for MCF-7 IRS-1 cells that had been cultured in the presence of doxycycline, 20 ng/ml; 2.6 nM and above for MCF-7 IRS-2 cells that had been cultured in the absence of doxycycline and 2 ng/ml; 0.26 nM and above for MCF-7 IRS-2 cells that had been cultured in the presence of doxycycline (ANOVA, $P<0.05$).

half-maximal stimulation of cell migration was reduced (Fig. 4B and C). This shows that increased IRS-1 and IRS-2 expression increases sensitivity to IGF-1 stimulated migration. For MCF-7 IRS-1 cells, this was approximately sixfold (from 6.5 to 1.1 ng/ml) whereas for MCF-7 IRS-2 cells it was approximately tenfold (from 7 to 0.7 ng/ml).

Effect of increased expression of IRS-1 and IRS-2 on MDA-MB-231 cell migration

The effect of increasing the expression of IRS-1 and IRS-2 on IGF-1 stimulated cell migration was tested by culturing the transfected MDA-MB-231 cells for 48 h in 2 µg/ml doxycycline. Doxycycline had no effect on the parental MDA-MB-231 Tet-on cells (Fig. 5A). Cell migration was increased approximately tenfold and the concentration of IGF-1 required for half-maximal cell migration was 12 ng/ml in the absence and presence of doxycycline.

By contrast, the response of MDA-MB-231 IRS-1 (Fig. 5B) and MDA-MB-231 IRS-2 (Fig. 5C) cells to IGF-1 was altered by prior treatment with doxycycline. The number of migrated cells at higher concentrations of IGF-1 was not altered but the concentration of IGF-1 required for half-maximal stimulation of cell migration was reduced. This shows that, as for MCF-7 cells, IRS-1 and IRS-2 expression increases sensitivity to IGF-1. For MDA-MB-231 IRS-1 cells this was approximately sixfold (from 17 to 3 ng/ml) whereas for MDA-MB-231 IRS-2 cells it was ~17-fold (from 10.5 to 0.6 ng/ml).

Effect of decreased expression of IRS-1 and IRS-2 on MCF-7 and MDA-MB-231 cell migration

The effect of decreasing the expression of the type I IGF receptor, IRS-1 and IRS-2 on IGF-1 stimulated cell migration was tested in MCF-7 and MDA-MB-231 cells in which expression had been knocked down by transfection of the cells with siRNA plasmids as

Figure 5 Effect of increased expression of IRS-1 and IRS-2 on IGF-1 stimulated cell migration in MDA-MB-231 cells. MDA-MB-231 cells transfected with pTRE2 (A), pTRE2 IRS-1 (B) and pTRE2 IRS-2 (C) were cultured in the absence and presence of doxycycline and the migration in response to different concentrations of IGF-1 was measured as described in the legend to Fig. 2. Circles show cell migration after culture in the absence of doxycycline, whereas triangles show cell migration after culture in the presence of doxycycline. The bars show the s.e.m. of at least three experiments. Asterisks show the concentrations at which migration is statistically significantly higher in the cells that had been cultured in the presence of doxycycline (*t*-test, $P=0.04$ at 5 ng/ml; 0.65 nM for MDA-MB-231 IRS-1 and $P=0.047$ at 2 ng/ml; 0.26 nM, $P=0.015$ at 5 ng/ml; 0.65 nM and $P=0.014$ at 10 ng/ml; 1.3 nM for MDA-MB-231 IRS-2 than in cells that had been cultured in the absence of doxycycline. The migration was statistically higher in cells stimulated with IGF-1 than in unstimulated cells at concentrations of 10 ng/ml; 1.3 nM and above for MDA-MB-231 cells that had been cultured in the absence and presence of doxycycline, 50 ng/ml; 6.5 nM for MDA-MB-231 IRS-1 cells that had been cultured in the absence of doxycycline, 20 ng/ml; 2.6 nM and above for MDA-MB-231 IRS-1 cells that had been cultured in the presence of doxycycline, 20 ng/ml; 2.6 nM and above for MDA-MB-231 IRS-2 cells that had been cultured in the absence of doxycycline and 2 ng/ml; 0.26 nM and above for MDA-MB-231 IRS-2 cells that had been cultured in the presence of doxycycline (ANOVA, $P<0.05$).

Figure 6 Expression of the type I IGF receptor, IRS-1 and IRS-2 in breast cancer cells transfected with pKD-IGF-IR; pKD-IRS1 or pKD-IRS2. MCF-7 and MDA-MB-231 cells were transfected with siRNA plasmids: pKD-IGF-IR-v2 (IGF-IR); pKD-IRS1-v3 (IRS-1) or pKD-IRS2-v4 (IRS-2; Upstate) in the presence of FuGENE HD transfection reagent (Roche) as described in the Materials and methods. The expression of the type I IGF receptor, IRS-1 and IRS-2 was measured in the transfected MCF-7 and MDA-MB-231 cells following culture under the conditions used during preparation of the cells for the migration experiments, as described in the Materials and methods. The cells were lysed and protein extracts prepared as described in the Materials and methods. The amounts of the type I IGF receptor, IRS-1 and IRS-2 proteins were measured by western transfer analysis as described in the Materials and methods. Aliquots of 5 μ g protein were electrophoresed on gradient polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with a 1:3000 dilution of specific type I IGF receptor antibody (Cell Signalling, 3027), a 1:4000 dilution of specific IRS-1 antibody (Upstate, 06-248), a 1:500 dilution of specific IRS-2 antibody (Santa Cruz, sc-1555-R) or a 1:20 000 dilution of specific GAPDH antibody (Santa Cruz, sc-25778) overnight at 4 °C. The filters were washed and incubated with HRP-conjugated goat-anti-rabbit antibody and the proteins detected using enhanced chemiluminescence (Western Dura Super Signal extended duration substrate, Pierce).

described in the Materials and methods. Representative examples of the knockdown achieved in the cells after transfection and subsequent culture in the withdrawal medium prior to examination of the migratory response to IGF-1 are shown in Fig. 6. The migratory response to all concentrations of IGF-1 was reduced significantly in MDA-MB-231 and MCF-7 cells in which the endogenous expression of the type I IGF receptor had been lowered (Fig. 7). Decreased expression of endogenous IRS-1 or IRS-2 reduced migration stimulated by 11 and 100 ng/ml IGF-1 in MDA-MB-231 cells and by 100 ng/ml (13 nM) IGF-1 in MCF-7 cells (Fig. 7). The greater effect of reducing expression of the type I IGF receptor than either IRS-1 or IRS-2 led us to test the effect of decreasing the expression of IRS-1 and IRS-2 simultaneously. The stimulation of migration by different concentrations of IGF-1 was analysed in MCF-7 cells in which both IRS-1 and IRS-2 expression had been knocked down (Fig. 7C). There was a significant reduction in the migration stimulated by 3.5, 11 and 35 ng/ml IGF-1 (0.45–4.55 nM) in cells with reduced IRS-1 and IRS-2 expression.

Discussion

The IGF signal transduction system has several properties that are compatible with a role in the progression of cancer. IGFs are mitogenic for many cell types, inhibit apoptosis and stimulate cell migration. These effects of IGFs on cancer cells are of increasing interest and are one of the reasons why the IGF signal transduction pathway has captured the imagination of the pharmaceutical industry as a therapeutic target for the treatment of cancer (Baserga 2004, Hofmann & Garcia-Echeverria 2005, Sachdev & Yee 2006).

Our results show that elevated expression of both IRS-1 and IRS-2 increases the sensitivity of the migration of both MCF-7 and MDA-MB-231 cells to lower IGF-1 concentrations but does not increase migration at higher IGF concentrations. This implies that IRS-1 and IRS-2 are important molecules in controlling breast cancer cell migration and invasion. The knockdown experiments demonstrate that IRS-1 and IRS-2 expressed by breast tumour cells contribute

to the migratory response of the breast cancer cells, and reinforce the conclusions based on the experiments in which IRS-1 and IRS-2 expression is increased in breast cancer cells.

There have been very few studies on the pathways and molecules downstream of the type I IGF receptor that mediate cell migration and invasion. Bartucci

et al. (2001) implicated the PI3 kinase/Akt pathway, whereas Mira *et al.* (2001) have emphasised the role of cytokines and shown that IGF-1 increases expression of the cytokine CCL5 and causes a redistribution of its receptor CCR5 to the leading edge of migratory cells.

It is possible that the migratory effects of IGFs are mediated through IRS-1 and IRS-2 as these are immediate downstream signalling proteins from the type I IGF receptor and are phosphorylated rapidly following treatment of cells with IGF-1. Byron *et al.* (2006) have suggested recently that IRS-1 mediates the mitogenic effects of IGFs, whereas IRS-2 mediates the mitogenic effects on the basis of experiments in which T47D cells that do not express IRS-1 or IRS-2 were transfected with IRS-1 and IRS-2 expression plasmids. These results differ from ours in that we find that higher expression of both IRS-1 and IRS-2 increases the migration of two breast cancer cell lines stimulated by lower IGF concentrations. Increased IRS-1 and IRS-2 did not, however, increase significantly cell migration stimulated by higher IGF-1 concentrations. The different conclusions may be attributed to one of the several factors including the use of different cell lines, different migration assays (Boyden chamber versus gold particle assay). More interestingly, they may result from the use of a single relatively high IGF-1 concentration by Byron *et al.* (2006), as at the concentration of 38.5 ng/ml (5 nM) IGF-1 used by these authors, we found near maximal stimulation of both untransfected and transfected cells. An alternative explanation is that IRS-1 requires the presence of IRS-2 to stimulate cell migration.

The observation that the amounts of both IRS-1 and IRS-2 influence the sensitivity of cell migration to IGFs suggests that both of these proteins are present in amounts that limit the sensitivity of the response and are therefore the key proteins in the signal transduction

Figure 7 Effect of decreased expression of type I IGF receptor, IRS-1 and IRS-2 on IGF-1 stimulated cell migration in MCF-7 and MDA-MB-231 cells. MCF-7 (A and C) and MDA-MB-231 (B) cells were transfected with control vector or siRNA plasmids: pKD-IGF-IR-v2; pKD-IRS1-v3 or pKD-IRS2-v4 and the migration stimulated by different concentrations of IGF-1 between 0 and 100 ng/ml (0–13 nM) was measured in triplicate experiments as described in the Materials and methods. Migration of control transfected cells (control) and cells transfected with siRNA plasmids: pKD-IGF-IR-v2 (IGF-IR); pKD-IRS1-v3 (IRS-1) or pKD-IRS2-v4 (IRS-2) are shown (A and B). The bars show the S.E.M. Open circles show the migration stimulated by different concentrations of IGF-1 in control transfected cells and filled circles the migration of cells transfected with pKD-IRS1-v3 and pKD-IRS2-v4 (C). The bars show the S.E.M. Asterisks show the IGF-1 concentrations at which migration is statistically significantly lower in the cells transfected with the siRNA plasmids than in the control cells (*t*-test, $P < 0.05$).

pathway controlling the mitogenic response to IGFs. It also suggests that these two proteins can substitute for each other in the pathway although their relative importance remains to be defined. Increasing the expression of IRS-1 and IRS-2 to very high levels may lead to super-sensitivity to IGFs which may be interpreted as IGF resistance in a similar way that oestrogen resistant breast cancer cells have been reported to be exquisitely sensitive to low concentrations of oestrogen (Chan *et al.* 2002). We have reported previously that oestrogens increase the expression of the type I IGF receptor (Stewart *et al.* 1990) and that this alters the sensitivity of the proliferative response to IGF-1 but not the magnitude of the response (Daws *et al.* 1996). This observation is consistent with the view that type I IGF receptor levels as well as the levels of IRS-1 and IRS-2 are important in controlling cellular responses to IGF-1.

We have observed previously that oestrogen increases the expression of IRS-1 (Molloy *et al.* 2000) and have suggested that this may be the mechanism by which oestrogens increase the response of breast cancer cells to IGFs (Molloy *et al.* 2000). The observation that IRS-1 and IRS-2 are important in the migratory response to IGF-1 may suggest that oestrogens could contribute to tumour progression through their effects on IRS-1 and IRS-2 expression. IRS-1 may be as important as IRS-2 as although increased levels of IRS-2 have a more pronounced effect on the sensitivity of cell migration to IGF-1, oestrogen induces IRS-1 expression to a greater extent than IRS-2. The importance of IRS-1 and IRS-2 in mediating IGF stimulated migration is reinforced by the demonstration that their knockdown decreases the migration stimulated by IGF-1 in MCF-7 and MDA-MB-231 cells. Taken together, our results suggest that the levels of endogenous expression of the type I IGF receptor, IRS-1 and IRS-2, which are regulated by oestrogen in oestrogen-responsive breast cancer cells, affect the mitogenic response to IGFs. This implies that the increased expression induced in the presence of oestrogen will impact significantly on the invasive behaviour of breast tumour cells.

There is increasing interest in cancer treatments that reduce the establishment and growth of metastatic deposits and inhibitors of the IGF signal transduction pathway may be effective in this clinical setting. These inhibitors would complement other inhibitors that have been identified such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, which have proven effectiveness in a murine model of pulmonary metastasis of breast cancer (Choi *et al.* 2006), vascular endothelial growth factor (VEGF)

inhibitors, which have been developed to inhibit vascularisation of secondary tumours (Mazitschek & Giannis 2004), and CXCR4 inhibitors, which inhibit the migratory effects of CXCL12 (Lee *et al.* 2004).

Inhibitors of the IGF system, all of which target the type I IGF receptor, have now reached the stage of clinical evaluation. The strategies used to date include neutralising antibodies, dominant negative receptor mutants, inhibitors of autophosphorylation and protein tyrosine kinase activity. There have been no reports of agents targeted against IRS proteins, apart from dominant negative mutants that lack the carboxy-terminus (Tanaka & Wands 1996) or all tyrosine residues (White 2002). Inhibitors of IRS-1 and IRS-2 may refine the specificity and reduce the side effects of IGF targeted therapy.

In conclusion, both IRS-1 and IRS-2 levels control the migratory response of breast cancer cells to IGF-1 and may, therefore, be key molecules in determining breast cancer spread. The IRS proteins may be at the convergence point of the effects of oestrogens and IGFs on cell proliferation and migration and could be therapeutic targets in oestrogen-responsive and non-responsive breast cancer cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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