Studies of epidermal growth factor receptor inhibition in breast cancer

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Abstract

Until recently, there has been little knowledge on the growth control of oestrogen receptor (ER)-negative ductal carcinoma *in situ* (DCIS) and invasive breast cancer. The recent development of DCIS models, such as transgenic mice, cell-line xenograft models and, importantly, *in vivo* human DCIS xenograft models has facilitated the investigation and understanding of the control of growth of early pre-invasive breast lesions.

Recent studies have shown that ER-negative DCIS, unlike ER-positive DCIS, is hormone independent and does not respond to anti-oestrogen treatment. Moreover, DCIS of the comedo type utilises type I tyrosine kinase growth factors, such as epidermal growth factor receptor (EGFR) and c-erbB-2, in receptor signalling for growth. New data underscore the importance of EGFR as the major modulating growth factor receptor in the control of proliferation in the breast.

Pre-clinical studies performed on human DCIS xenografts in nude mice suggest a potential role for EGFR tyrosine kinase inhibitors (EGFR-TKIs). More specifically, ZD1839, a novel orally active and selective EGFR-TKI, has been shown to produce a response in DCIS through a decrease in epithelial proliferation. These findings have enhanced our knowledge of signal transduction pathways in cancer and indicate that tyrosine kinase blockade of EGFR has potential for the treatment and chemoprevention of DCIS.

It is hoped that further advances in this area and evaluation of EGFR-TKIs in Phase II/III clinical trials will allow their therapeutic potential as anticancer agents to be appreciated.

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Introduction

Earlier diagnosis by mammographic screening has resulted in an increase in the incidence of breast cancer. Ductal carcinoma *in situ* (DCIS) is a pre-invasive breast lesion that accounts for 30% of cases of screen-detected breast cancer (Ernster *et al.* 1996). Untreated DCIS progresses to invasive breast cancer in 25–30% of patients. Greater use of breast conserving surgery and concern in preventing the development of breast cancer with adjuvant anti-oestrogens or other drugs has led to an increasing interest in the mechanism underlying the development of DCIS (Fisher *et al.* 1998, Ettinger *et al.* 1999).

The majority (70%) of DCIS specimens identified by mammographic microcalcification are of the high-grade comedo type, associated with high proliferative rate, negative oestrogen receptor (ER) status, expression of epidermal growth factor receptor (EGFR), and Neu/c-erbB-2 oncoprotein over-expression (Bobrow & Millis 1995). ER-negative DCIS, unlike ER-positive DCIS, is expected to

be hormone independent and, therefore, unlikely to respond to treatment with anti-oestrogens.

Human ductal carcinoma *in situ* xenograft model

The absence of suitable cell lines for DCIS has led to the development of *in vivo* human DCIS xenograft models in which to study the growth of early proliferative breast lesions. The use of normal human breast tissue xenografts implanted into athymic nude mice demonstrated that more than 95% of xenografts could be retrieved (Laidlaw *et al.* 1995). In the same study, oestrogen was shown to increase epithelial proliferation and to induce the expression of progesterone receptor (PR), unlike progesterone, which exhibited no effects.

To determine the effects of oestrogen supplementation on cell proliferation in transplanted DCIS, a novel *in vivo* animal model was developed, in which human breast DCIS xenografts could be subjected to hormonal manipulation. In

this model, xenografts of breast tissue (approximately $2 \times 2 \times 1$ mm in size) taken from areas of mammographic microcalcification containing histologically verified DCIS in women undergoing mastectomy were implanted into athymic mice (Holland *et al.* 1997). More than half (58%) of the xenografts processed for implantation, and subsequently 22.3% of all xenografts retrieved from the animals, contained foci of DCIS. The median retrieval of DCIS xenografts at the end of each experiment was 94.5% (range 48–100%). Xenografts from the majority of specimens (83%) survived for up to 56 days in the mice and maintained good architectural and cellular preservation.

In the same study, in order to determine the degree of oestrogen dependence for DCIS growth in relation to steroid receptor status, 50% of the mice received implants containing oestrogen, giving a concentration of oestradiol of approximately 1400 pmol/l, compared with concentrations of 125 pmol/l in unsupplemented mice. Levels of cell proliferation in the xenografts recovered after 14, 28, 42 or 56 days in the mice were measured. A significant increase in epithelial proliferation and apoptosis was observed in ER-positive DCIS stimulated by oestrogen ($P < 0.001$), unlike ER-negative DCIS, which showed no change in proliferation or apoptosis in response to oestrogen treatment ($P = 0.89$). The investigators concluded that ER-negative, comedo DCIS lesions appear to be largely oestrogen independent, and that the drive for cell proliferation must be provided by growth factors other than oestrogen. Therefore, anti-oestrogen therapy is not expected to benefit patients with comedo DCIS, whereas ER-positive DCIS responds by a decrease in proliferation or an increase in cell death, or both.

Anti-oestrogen therapy

To aid further investigation into the effects of anti-oestrogen treatment on DCIS, the same model was used to expose human DCIS xenografts to pure anti-oestrogen therapy and to examine its effects on rates of cell proliferation and apoptotic cell death (Gandhi *et al.* 2000). The apoptotic index was found to be higher in the day 0 specimens (i.e. tissue representative of that excised from patients at the time of surgery) of ER-negative DCIS lesions than in ER-positive DCIS lesions (1.47% (range 1.21–1.77%) compared with 0.32% (range 0.22–0.45%); $P < 0.001$) (Fig. 1). Similarly, cell proliferation, as measured by the labelling index of the Ki67 nuclear antigen in immunohistochemical staining, was greater in ER-negative DCIS than in ER-positive DCIS lesions (20.6% (range 18.5–22.9%) compared with 3.1% (range 2.2–4.2%); $P < 0.001$). Apoptotic index and labelling index values within ER-negative DCIS did not differ between xenografts exposed to oestrogen or anti-oestrogen treatment compared with controls. In contrast, ER-positive DCIS showed an increase in apoptotic index and labelling index, as expected after 14 days of oestrogen therapy. Anti-oestrogen

therapy with ICI 182,780 (Faslodex; AstraZeneca, Alderley Edge, Cheshire, UK) of ER-positive DCIS did not affect the labelling index, but resulted in a greater apoptotic index than was observed in controls. ICI 182,780 also significantly inhibited the expression of PR, an oestrogen-regulated protein, in ER-positive DCIS, as confirmed by immunohistochemical assessment. PR protein expression in day 0 ER-positive xenografts was 39.25% (range 32.14–47.94%), compared with 46.53% (range 41.68–51.94%) after 28 days of oestrogen treatment and 18.17% (range 17.99–18.24%) after 28 days of ICI 182,780 treatment ($P < 0.001$). Therefore, ER-positive DCIS proliferates under hormonal influences and responds to anti-oestrogen therapy, unlike ER-negative DCIS, which is oestrogen independent.

Relevance of anti-oestrogen therapy findings to the clinical situation

In two studies (Fisher *et al.* 1999, George *et al.* 2000), patients were allocated randomly to groups to receive adjuvant tamoxifen or placebo after surgical therapy. The National Surgical Adjuvant Breast and Bowel Project B-24 study (Fisher *et al.* 1999), in which women were allocated randomly to groups after breast-conserving surgery and localised radiotherapy, demonstrated that women receiving tamoxifen had fewer breast cancer events at 5 years than women receiving placebo (8.2% compared with 13.4%; $P = 0.0009$). Although a reduced incidence of ipsilateral invasive breast cancer was observed, no reduction in ipsilateral DCIS was detected. In addition, one in three women were reported to be unable to complete the course of tamoxifen treatment because of drug-related side effects or 'personal reasons'. There was also an excess of endometrial cancer and thromboembolic events in the tamoxifen group compared with controls. These adverse events in women with unresponsive DCIS (such as ER-negative DCIS) assume greater clinical concern. It is worth noting that around one-third of the women in this trial were younger than 50 years and the majority of the benefits of tamoxifen were found in this age group.

In the UK DCIS trial (George *et al.* 2000), which predominantly (93%) consisted of women aged 50 years or more, patients were allocated randomly to groups to receive radiotherapy and tamoxifen, or either intervention alone. Tamoxifen had no effect in the presence of radiotherapy, although a small but non-significant benefit was detected with tamoxifen therapy in the absence of radiotherapy.

Therefore, although tamoxifen appears to be beneficial in younger women, its value in older women (over 50 years of age) treated for DCIS remains unclear. The mechanism by which tamoxifen reduces recurrent ipsilateral invasive cancer in younger women may relate to the potential of this drug to inhibit the effects of endogenous oestrogen in the breast, rather than to exert direct effects on DCIS.

Figure 1 Apoptotic cell death in ER-negative (ER-; solid symbols, solid line) and ER-positive (ER+; open symbols, dotted line) DCIS. Apoptosis was assessed by morphological assessment of haematoxylin and eosin sections of xenografts retrieved from mice exposed to 17β-oestradiol, anti-oestrogen, or a control vehicle oil. An apoptotic index was calculated by noting the number of cells showing morphological evidence of apoptotic cell death as a percentage of total number of cells counted in each retrieved xenograft containing DCIS. Results are presented as geometric mean values with their 95% confidence intervals. The number of xenografts used for each time point is presented beneath the chart for ER-negative and ER-positive DCIS. At day 0 ER-negative DCIS displayed a much greater apoptotic index than ER-positive DCIS. In both subtypes the apoptotic index did not change significantly in the 14-day pre-treatment period; treatment was commenced on day 14. Hormonal manipulation of ER-negative DCIS had no effect on apoptotic cell death compared with control or pre-treatment xenografts. ER-positive DCIS displayed a significant increase in cell death when exposed to 17β-oestradiol or anti-oestrogen therapy. (Reproduced with permission from Gandhi *et al.* 2000.)

Role of growth factor receptors in ER-negative ductal carcinoma *in situ*

ER-negative DCIS has been shown to express type 1 tyrosine kinase growth factor receptors, such as EGFR and c-erbB-2 (Hanna *et al.* 1990, Pavelic *et al.* 1992, Bobrow & Millis 1995, Gompel *et al.* 1996, Robertson *et al.* 1996). Both EGFR and c-erbB-2 have an extracellular receptor binding domain and a cytoplasmic tyrosine kinase domain. On attachment of a ligand, the receptors dimerise, resulting in homodimerisation or heterodimerisation (Wells 1999). Dimerisation results in tyrosine kinase autophosphorylation, which in turn activates a number of intracellular signalling pathways, including the mitogen-activated protein (MAP) kinase signalling pathway (Fig. 2), leading to cell proliferation and prevention of apoptosis (Ullrich & Schlessinger 1990, Dougall *et al.* 1993, Muthuswamy *et al.* 1994, Baselga & Mendelsohn 1997).

EGFR is a 170 kDa transmembrane glycoprotein expressed on many epithelial cells (Gompel *et al.* 1996). It is activated by at least six ligands, including epidermal growth factor (EGF), transforming growth factor (TGF)-α and heparin-binding EGF. In a variety of tumours, up-regulated EGFR signalling has been correlated with progression to invasion and metastasis (Wells 1999).

It has been shown that 10–15% of luminal epithelial cells within the normal breast express steroid hormone receptors; however, dividing cells are steroid hormone receptor-negative (Clarke *et al.* 1997). As human mammary epithelial cells, which are steroid hormone receptor-negative, respond to EGF and insulin-like growth factor (IGF-I), it is likely that growth in the breast is largely controlled by stimulation of these two growth factor receptors (Price *et al.* 1990, Silverstein 1998).

Stimulation of the IGF-I receptor leads to release of TGF-α from the cell, which transactivates the EGFR in the same cell in an autocrine manner (Roudabush *et al.* 2000). Therefore, evidence is accumulating that the EGFR is the main receptor pathway associated with epithelial proliferation in the human breast (Gompel *et al.* 1996).

c-erbB-2 is a 185 kDa transmembrane tyrosine kinase with considerable homology to EGFR, and for which a ligand has not yet been identified. The presence of c-erbB-2 may allow more frequent formation of c-erbB-2 heterodimers and, therefore, augmentation of EGFR cell-surface signalling in the MAP kinase pathway, leading to increased proliferation (Ullrich & Schlessinger 1990, Dougall *et al.* 1993, Muthuswamy *et al.* 1994, Wells 1999).

Heterodimerisation of c-erbB-2 with c-erbB-3 is also a possible explanation for tumour development. Tumour

Figure 2 Schematic representation of EGFR signal transduction and its downstream effects. K = kinase; pY = phosphotyrosine; R = receptor; PI3-K, phosphatidylinositol 3-kinase; SOS, Son of Sevenless; GRB2, growth factor receptor-bound 2; MEK, MAP extracellular signal-regulated kinase; MAPK, MAP kinase; PTEN, phosphatase and tensin homologue deleted from chromosome 10; AKT, AKR mouse transforming; STAT, signal transducer and activator of transcription. (Adapted with permission from Baselega *et al.* 2000.)

progression in mice containing activated c-erbB-2 oncogenes is associated with increased concentrations of phosphorylated c-erbB-2 and c-erbB-3 (Siegel *et al.* 1999, Dankort & Muller 2000). Moreover, frequent co-expression of transcripts for both c-erbB-2 and c-erbB-3 has recently been found in a series of primary breast tumours (Siegel *et al.* 1999). Therefore, metastasis may result from c-erbB-2/c-erbB-3 heterodimers, although the low expression of c-erbB-2 in normal breast suggests that DCIS development involves EGFR rather than c-erbB-2 (Gompel *et al.* 1996).

In MCF-7 cells transfected with c-erbB-2 and injected into nude mice to form tumours, resistance to tamoxifen therapy was seen, but the tumours maintained a growth-inhibitory response to oestrogen withdrawal (Benz *et al.* 1992). Interestingly, c-erbB-2 monoclonal antibody therapy has been shown to produce tumour responses in women with metastatic breast cancer (Cobleigh *et al.* 1999). Moreover, tyrosine kinase blockade of the EGFR inhibits proliferation or induces apoptosis (or both) *in vitro* and *in vivo* in breast, ovary, lung, colonic and gastric cell lines, with generally mild and reversible toxicity (Woodburn *et al.* 1998, Ciardiello *et al.* 2001).

Growth factor receptor inhibitors decrease epithelial proliferation in ductal carcinoma *in situ*

A recent study has investigated the effects of c-erbB-2 monoclonal antibody therapy and EGFR tyrosine kinase

inhibition on epithelial proliferation and apoptosis in DCIS (Chan *et al.* 2001), using the DCIS xenograft model described previously (Holland *et al.* 1997, Gandhi *et al.* 2000).

The effects of 4D5 monoclonal antibody directed against an epitope on the extracellular domain of c-erbB-2 (trastuzumab) were investigated. Positive control experiments were conducted in which SKOV3 human ovarian carcinoma cells were implanted into athymic nude mice and allowed to form tumours for 14 days. Intraperitoneal injection of 4D5 monoclonal antibody resulted in significant tumour growth inhibition in SKOV3 cells after 21 days of treatment ($P < 0.01$). Of seven DCIS xenograft experiments in which 4D5 monoclonal antibody was used, five (71%) were immunohistochemically c-erbB-2-positive, with all seven DCIS specimens being of the comedo type and ER-negative.

After 28 days of treatment with anti-c-erbB-2 monoclonal antibody, the median labelling index in c-erbB-2-positive DCIS was 24.5 (interquartile range 11.6–36.8), compared with 19.9 (interquartile range 15.0–35.2) in the control group ($P = 0.88$). The median apoptotic index was 0.72 (interquartile range 0.40–1.07) in the 4D5-treated group, compared with 0.96 (interquartile range 0.83–1.77) in the control group ($P = 0.14$). Therefore, non-significant effects on epithelial proliferation and apoptosis were observed with 4D5 monoclonal antibody in ER-negative DCIS xenografts. Similar data were obtained with c-erbB-2-negative 4D5-treated xenografts compared with control DCIS xenografts.

The lack of response may relate to the intact basement membrane of DCIS, which may present a physical barrier to the large molecule of 4D5 antibody, thus precluding its penetration into the DCIS basement membrane and its interaction with the c-erbB-2 receptor (Holland *et al.* 1997). Alternatively, c-erbB-2 oncoprotein may not be the main receptor driving epithelial proliferation in DCIS (Robertson *et al.* 1996).

In the same study, the effects of the novel EGFR tyrosine kinase inhibitor (EGFR-TKI) ZD1839 (Iressa; AstraZeneca) on epithelial proliferation and apoptosis were investigated. ZD1839 has a molecular weight of 446 kDa and, therefore, has a greater likelihood of penetrating through the basement membrane of DCIS. It is an orally active and selective EGFR-TKI, with 100 times greater specificity for EGFR tyrosine kinase than c-erbB-2 tyrosine kinase, and 1000 times greater specificity for EGFR tyrosine kinase than c-flt-1 tyrosine kinase (Woodburn *et al.* 2000).

Positive control experiments with the human breast carcinoma cell line MDAMB231 demonstrated a significant inhibition of tumour volume growth after 7 days of treatment with ZD1839 ($P < 0.01$). The proliferation index (as judged by Ki67 staining) was significantly decreased by ZD1839 compared with that in control-treated tumours. Treatment with ZD1839 (but not vehicle control) decreased phosphorylated EGFR and MAP kinase activity in the tumours. Almost all (10/11; 91%) of DCIS xenograft experiments in which ZD1839 was used were immunohistochemically positive for EGFR. Eight of ten EGFR-positive DCIS xenografts were ER-negative.

After 14 days of treatment (day 28) with ZD1839 (100–200 mg/kg), a significant decrease in proliferation was detected compared with controls ($P < 0.01$) (Fig. 3). This decrease was maintained when treatment was extended to 28 days (day 42) at ZD1839 75 mg/kg ($P < 0.05$). The median apoptotic index increased after 7 days of treatment (day 21) in the ZD1839-treated group compared with controls. The labelling index and apoptotic index data were similar to those obtained with EGFR-negative DCIS xenografts and ER-positive DCIS treated with ZD1839.

These data clearly show that EGFR tyrosine kinase inhibition with ZD1839 decreases proliferation and activates apoptosis in DCIS *in vivo*. These findings suggest that ZD1839 is a potential agent for the treatment and chemoprevention of DCIS. In support of these observations are data from a recent *in vitro* study showing a significantly marked decrease in proliferation with ZD1839 in MCF7 breast cancer cells, thereby strongly suggesting that cell proliferation in breast cancer is driven via EGFR signalling pathways (Gee *et al.* 2001, McClelland *et al.* 2001).

It has previously been shown that the preferred heterodimerisation partner for all type I tyrosine kinase receptors, including EGFR, is c-erbB-2 (Graus-Porta *et al.* 1997). It is possible that ZD1839 may inhibit

Figure 3 Effect of ZD1839 (200 mg/kg daily) on epithelial proliferation (Ki67 labelling index, LI) of DCIS retrieved xenografts. ZD1839 or vehicle was given from day 14 onwards. Horizontal lines represent median values and boxes denote the interquartile ranges. The number of xenografts used for each time point is presented beneath the chart. A significant decrease in proliferation was detected in the ZD1839 group after 14 days of treatment (day 28) compared with controls ($*P < 0.01$, Mann-Whitney *U* test).

EGFR-mediated phosphorylation and activation of c-erbB-2 within EGFR-c-erbB-2 heterodimers, resulting in decreased proliferation and increased apoptosis. In support of this we have found that ZD1839 inhibits the proliferation of breast cancer cell lines that over-express c-erbB-2 (Fig. 4 and Anderson *et al.* 2001).

Figure 4 Anti-proliferative effects of ZD1839 in EGFR-positive cancer cells with varying levels of c-erbB-2 expression. Low-density cell cultures growing in medium containing 10% foetal calf serum were treated for 48 h with ZD1839 (1 μ mol/l). Cell numbers were then counted and related to cultures treated with drug vehicle (0.1% DMSO). All values are means $\pm$ S.E.M. from at least three independent experiments. The relative levels of expression of EGFR and erbB-2 (+++, high; ++, medium; +, low) were determined by immunoblotting.

Importantly, despite the fact that EGFR is expressed in many tissues, no drug-related toxicity was detected in the mice treated with ZD1839 (Chan *et al.* 2001). Phase I clinical trials of ZD1839 have shown that its toxicity is manageable, with the most common side effects including acne-like rash, diarrhoea, nausea and vomiting (Baselga *et al.* 2000, Ferry *et al.* 2000, Goss *et al.* 2000; Negoro *et al.* 2001). Phase II and III clinical trials of this drug are currently in progress.

Discussion

Accumulating evidence on the expression and function of EGFR in normal breast and DCIS supports the concept that the major modulating growth factor receptor in murine and human breast is EGFR. It has been shown that, compared with the low expression of c-erbB-2, EGFR is widely expressed in normal breast tissue (Gompel *et al.* 1996). Moreover, a quantification analysis in the human breast cancer has indicated that, unlike c-erbB-2 expression, EGFR expression in tumours is correlated directly with bromodeoxyuridine labelling index, a surrogate measure of epithelial proliferation (Robertson *et al.* 1996). Both EGFR and c-erbB-2 are expressed in ER-negative, comedo-type DCIS (Bobrow & Millis 1995). EGFR is also expressed in ER-positive DCIS (Chan *et al.* 2001). Receptor signalling is facilitated through the interaction of EGFR and c-erbB-2 in the formation of heterodimers (Graus-Porta *et al.* 1997). The presence of increased EGF, TGF- α , and EGF-like ligands in DCIS, combined with the expression of EGFR and over-expression of c-erbB-2, may explain the markedly increased proliferation and apoptosis detected in ER-negative DCIS.

Pre-clinical studies performed on human DCIS xenografts in nude mice *in vivo* suggest a potential role for novel agents that inhibit intracellular signal transduction pathways. More specifically, the orally active and selective EGFR-TKI ZD1839 has been shown to exert dual action on proliferation and apoptosis in EGFR-positive DCIS (Chan *et al.* 2001). The anti-proliferative action of this drug against EGFR-positive cell lines that over-express c-erbB-2 suggests that co-expression of c-erbB2 in EGFR-positive DCIS may not diminish the effectiveness of ZD1839 against this category of cancer. Therefore, EGFR-TKIs may have the potential as adjuvant therapy for the treatment and chemoprevention of DCIS. These findings on the growth control of ER-negative DCIS support further pre-clinical and clinical investigation of these agents as a novel therapy in DCIS (George *et al.* 2000).

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