The majority of patients had lesions which could be completely excised and this will be heartening news for the UK trial of screen-detected DCIS [1]. The experience of the EORTC Breast Cancer Cooperative Group in trial 10853 was that only one-third of patients with DCIS were suitable for the trial, which compares wide local excision with wide local excision and external radiotherapy (50 Gy) [2]. The majority of the contributing centres to trial 10853 were treating symptomatic rather than screened women.

Silverstein has shown, as have most other studies that axillary clearance is not required, and that mastectomy reduces the risk of ipsilateral recurrence to almost zero [3–5]. Additionally, even when patients with smaller lesions are selected for breast conservation there will be an increased risk of recurrence of DCIS or progression to invasive disease. Whether this can be altered by radiotherapy still remains unanswered.

Large well-controlled trials will be needed to determine this. EORTC trial 10853 is still open, and has to date accrued 276 cases. Eligible patients will have had DCIS completely excised (confirmed after pathological examination of inked edges). No axillary dissection is performed and no radiation boost is given to the biopsy site. Getting more surgeons and their patients to participate in this trial will enable sub-group analysis to be conducted of the various histological variants of DCIS. Then, perhaps, the effect of radiotherapy on DCIS will become apparent.

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Papers

Tamoxifen Up-regulates c-*erb*B-2 Expression in Oestrogen-responsive Breast Cancer Cells *in vitro*

Susanna Antoniotti, Piera Maggiora, Claudio Dati and Michele De Bortoli

Expression of the c-*erb*B-2 proto-oncogene is inhibited by oestrogens in oestrogen-responsive human breast cancer cells, at both mRNA and protein level. Here we report that, where the regulation of c-*erb*B-2 is concerned, tamoxifen displays a full anti-oestrogenic activity, enhancing the expression of c-*erb*B-2 in oestrogen receptor-positive cells cultured with untreated fetal calf serum or reversing the inhibitory effect of added oestrogens. Meanwhile, tamoxifen strongly inhibited cell growth. Tamoxifen was inactive on both c-*erb*B-2 expression and growth of oestrogen receptor-negative cells. These results may have important implications to explain occasional failure of tamoxifen therapy in oestrogen receptor-positive breast cancers.
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INTRODUCTION

THE c-*erb*B-2 proto-oncogene (also called HER2/*neu*) encodes a 185 kD transmembrane tyrosine kinase (p185) [1], sharing a 50% homology with the epidermal growth factor receptor [2]. c-*erb*B-2 is frequently amplified in a variety of human adenocarcinomas and the resulting p185 overexpression is thought to confer a particular aggressiveness to the tumour. Human breast cancer has been extensively investigated and c-*erb*B-2 amplification and overexpression shown to be associated with early relapse and death [3, 4].

Work from our and other laboratories has demonstrated that the expression of c-*erb*B-2 in mammary cells is subjected to hormonal regulation [5, 6]. In particular, we have recently shown that oestrogens specifically inhibit c-*erb*B-2 expression in breast cancer cells [5]. Oestrogen receptor positive (ER+) breast cancers are commonly treated with endocrine therapies, mainly by the use of anti-oestrogenic drugs such as tamoxifen, which shows clear antimitogenic properties on oestrogen-dependent cells.

Therefore, we have investigated the effect of tamoxifen on c-

*erb*B-2 mRNA and protein expression in ER+ and oestrogen receptor negative (ER−) breast cancer cell lines.

MATERIALS AND METHODS

Cell culture

Human breast cancer cell lines T47D, ZR75.1 (ER+) and MDA.MB.231 (ER−) were used. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (T47D) or 10% (ZR75.1, MDA.MB.231) fetal calf serum (FCS), 4 mmol/l l-glutamine, 20 mmol/l HEPES buffer, pH 7.3 and 50 U/ml penicillin and 50 μg/ml streptomycin (complete medium, CM). Experiments were carried out both in this medium and in a medium devoid of oestrogenic activity (oestrogen-free medium, EFM) prepared by treating FCS with dextran-coated charcoal and by omitting phenol red from the formulation. 10 nmol/l 17β-oestradiol and 1 μmol/l 4-hydroxy-tamoxifen or tamoxifen citrate were added, when appropriate, in ethanol. For harvesting, cells were washed twice with ice-cold phosphate buffered saline (PBS), scraped, quickly pelleted at 4°C, frozen in liquid nitrogen and stored at −80°C.

Evaluation of cell growth was done both by measuring the DNA content of the cultures and by direct counting of viable cells with a haemocytometer.

p185 immunoblotting

Cell pellets were lysed in 20 mmol/l Tris, pH 7.4, 0.1 mol/l NaCl, 5 mmol/l $MgCl_2$, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1 mmol/l 2-mercaptoethanol and 2 U/ml Trasylol. Lysates were cleared by centrifugation at 800 g for 20 min at 4°C and stored at −80°C. 100 μg lysate protein was then analysed for p185 level by immunoblotting [5]. As a positive control, 20 μg total protein extracted from SKBR. 3 cells was used. Detection was done with a polyclonal antiserum recognizing the 13 aminoacid sequence at the C-terminus of the human c-*erb*B-2 protein [7].

RNA extraction and analysis

Total RNA was extracted from cell pellets by the guanidine–lithium chloride procedure [5]. 20 μg was separated on 1.2% agarose–formaldehyde gels and blotted to Hybond-*N* membranes (Amersham). 2 μg of total RNA from SKBR.3 cells was used as positive control. Blots were hybridised at 42°C in 50% formamide, with 1–2 × 10^6 cpm per ml of random priming ^{32}P-labeled probes, and washed in 0.1 × SSC, 0.1% SDS at 65°C. The human c-*erb*B-2 probe used was a 1.1 kbp *Bam* HI 5′ fragment from the pCER204 cDNA [2]. Quantitative control was provided by ethidium bromide staining of the gel and by rehybridising the filters to a glyceraldehyde-phosphate dehydrogenase probe [8].

RESULTS

Growth of T47D and ZR75.1 cells was largely dependent upon oestrogens; cell proliferation was greatly reduced by culturing the cells in EFM or in the presence of tamoxifen, as compared to the growth in "complete" medium (CM) or in the presence of added 17β-oestradiol. Table 1 shows the data of a typical experiment with T47D cells. Expression of c-*erb*B-2 was studied both at the mRNA and at the protein level. By northern blotting, we could detect a major 5.1 kD band, which represents the main c-*erb*B-2 mRNA [9]. Analysis of the protein extract by immunoblotting with the 21N antibody revealed a major 185–190 kD protein, corresponding to the p185 product of c-*erb*B-2 [7].

Table 1. Growth rate of T47D cells in various experimental conditions, relative to the growth rate in CM

Treatment	Growth (%)
CM	100
CM + 4-OH-tamoxifen	46
EFM	54
EFM + E2	89
EFM + 4-OH-tamoxifen	36
EFM + E2 + 4-OH-tamoxifen	39

Results refer to the increase of DNA content of the cultures over a 4-day treatment. Each value is the mean of triplicate cultures, with individual variations ranging from 10 to 20%.

The reduced growth rate in EFM accompanied an evident elevation of c-*erb*B-2 mRNA and protein levels (Fig. 1, (A) and (B), respectively compare lanes a and c). Subsequent addition of 17β-oestradiol resulted in down-regulation of c-*erb*B-2 mRNA and protein to the original levels (Fig. 1, lanes e), as previously observed [5]. 1 μmol/l 4-OH-tamoxifen was able to enhance c-*erb*B-2 expression in both CM and EFM (Fig. 1, lanes b and d) and also partly reversed the effect of 10 nM 17β-oestradiol added to EFM (Fig. 1, lanes f). Similar results were obtained with ZR75.1 cells. Figure 2 shows an immunoblot of p185 levels in ZR75.1 cells treated with 17β-oestradiol or 4-OH-tamoxifen. On the contrary, no effects of either oestrogens or anti-oestrogens were seen on both the growth rate and c-*erb*B-2 expression of the ER− human breast cancer cell line MDA.MB.231. Figure

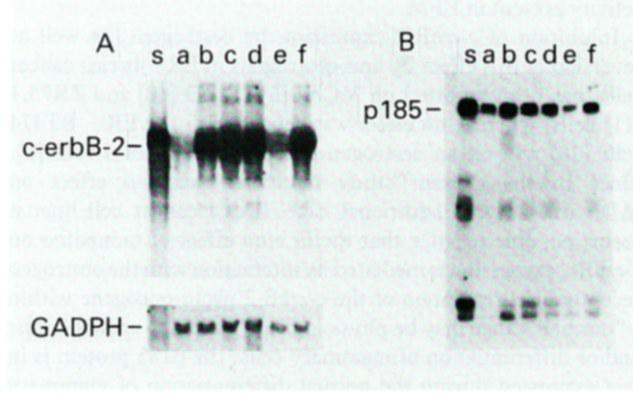

Fig. 1. Effects of 17β-estradiol (E2) and 4-OH-tamoxifen (OH-Tam) on c-*erb*B-2 mRNA and protein levels in T47D cells. T47D cells were grown in CM for 3 days, then treated as indicated for 4 days. (A) Northern blot of c-*erb*B-2 mRNA. Control hybridisation (lower) represents GAPDH mRNA. (B) Immunoblot of p185 level. Treatments were (a) CM; (b) CM + 4-OH-tamoxifen; (c) EFM; (d) EFM + 4-OH-tamoxifen; (e) EFM + E2; (f) EFM + E2 + 4-OH-tamoxifen (lane s) SKBR.3 positive control.

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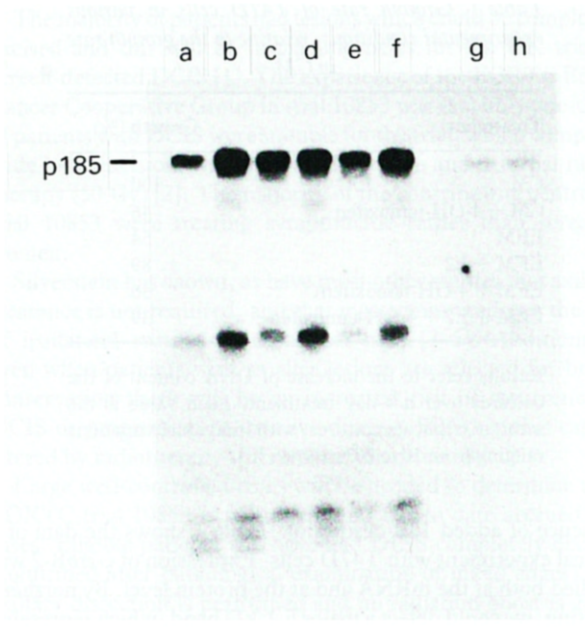

Fig. 2. Immunoblot of p185 expression in ER+ (ZR75.1) and ER− (MDA.MB.231) cell lines. Cell culture and treatments were as in Fig. 1. Lanes a–f: ZR75.1 cells, treatments as in Fig. 1, lanes a–f; lanes g, h: MDA.MB.231 cells, treated as ZR75.1, lanes a, b.

2 shows the p185 levels detected in MDA.MB.231 cells growing in CM, treated (lane h) or not (lane g) with 1 μmol/l 4-OH-tamoxifen for 3 days.

In all experimental conditions, the effects of tamoxifen citrate or 4-OH-tamoxifen were substantially identical.

DISCUSSION

Our data indicate that, in ER+ human breast cancer cells, tamoxifen up-regulates the expression of c-*erb*B-2 while inhibiting cell growth *in vitro*, showing, in this respect, a full anti-oestrogenic activity.

In our experimental conditions, both the inhibition of growth and the stimulation of c-*erb*B-2 expression obtained with tamoxifen were more pronounced than those we could obtain in EFM. This result is most likely due to some residual oestrogenic activity present in EFM.

Inhibition of c-*erb*B-2 expression by oestrogens, as well as reversion of this effect by anti-oestrogens in ER+ breast cancer cells, has been reported on MCF7 [6], T47D [10] and ZR75.1 [11] cells, whereas no effect was reported on the ER− BT474 cells [10] and on an oestrogen-resistant variant of T47D [6]. Since in the present study tamoxifen had no effect on MDA.MB.231, an additional ER− breast cancer cell line, it seems possible to infer that the *in vitro* effect of tamoxifen on c-*erb*B-2 expression is mediated by interaction with the oestrogen receptor. Up-regulation of the c-*erb*B-2 proto-oncogene within a "normal" range may be physiologically linked to growth arrest and/or differentiation of mammary cells; the p185 protein is in fact expressed during the normal differentiation of mammary tissues *in vivo* and *in vitro* and enhanced p185 levels are seen in breast cancer cells treated with DBcAMP or reaching confluence [ref. 5, and M. D.B. *et al.*]. In addition, transcription from the c-*erb*B-2 promoter is stimulated by retinoic acid, TPA and DBcAMP [12], which are inhibitors of breast cancer cell growth.

On the other hand, overexpression of c-*erb*B-2 due to gene amplification is thought to play a very relevant role in breast cancer progression. This is suggested not only by the many studies on the association of c-*erb*B-2 amplification with poor prognosis [4], but also by a number of more direct experimental evidences: transfection of a normal c-*erb*B-2 allele in constitutive expression vectors leads to neoplastic transformation [13, 14] and treatment with anti-p185 antibodies reduces the neoplastic potential of breast cancer [15] and other cells [16]. In addition, c-*erb*B-2 has been shown to induce mammary tumours in transgenic mice [17, 18]. Most likely, c-*erb*B-2 contributes to the malignant phenotype not merely by affecting proliferation, but also by increasing other properties of tumour cells, e.g. resistance to host defences [15].

The fact that a well-known therapeutic agent for breast cancer such tamoxifen increases the expression of c-*erb*B-2 *in vitro* seems paradoxical. However, the effects of tamoxifen *in vivo* may be more complex and widely different from what we have observed *in vitro*. LeRoy *et al.* have recently reported that ER− breast tumours from tamoxifen-treated patients showed lower c-*erb*B-2 mRNA levels than tumours from untreated patients [10]. Noteworthy, the decrease of c-*erb*B-2 expression after tamoxifen treatment was seen only in ER− tumours, but it was not reproducible *in vitro* on ER− cell lines, as also confirmed by data presented here. This implies that the *in vivo* effect of tamoxifen on c-*erb*B-2 expression in ER− breast tumour cells must be mediated by an unidentified endocrine or paracrine factor. It seems reasonable to conclude that, *in vivo*, tamoxifen exerts opposite effects on c-*erb*B-2 expression by two definite pathways: (1) a direct anti-oestrogenic effect, i.e. stimulation of c-*erb*B-2, by binding to oestrogen receptors in ER+ tumour cells; (2) inhibition of c-*erb*B-2 by affecting an unknown endocrine or paracrine axis, whose action may affect both ER+ and ER− tumour cells. In ER+ tumours, the resulting level of expression of c-*erb*B-2 will then reflect the balance of these opposite effects, depending on their relative strength.

Even though it is clear that the expression of c-*erb*B-2 is controlled *in vivo* by multiple factors, the enhancing effect of tamoxifen on c-*erb*B-2 expression *in vitro* reported here may have important implications. It can be hypothesised that, under circumstances that need to be investigated, tamoxifen can induce very high p185 levels in ER+ tumours carrying an amplified c-*erb*B-2 gene. In this eventuality, the antimitogenic effects of tamoxifen will be counterbalanced by the increased oncogenic potential associated with c-*erb*B-2 overexpression. This hypothesis, if true, may contribute to explain recent evidences that breast cancers with amplified c-*erb*B-2 do not respond favourably to anti-oestrogen therapy. In fact, Wright *et al.* have reported that most of breast cancer patients who did not respond to tamoxifen had p185-positive tumours [19] and Borg *et al.* found that c-*erb*B-2 amplification was correlated to poor prognosis mainly in the patient group receiving adjuvant therapy [20]. In addition, several studies have shown that c-*erb*B-2 amplification is associated with poor prognosis mainly in steroid receptor-positive tumours [21–24].

Further *in vitro* and *in vivo* experiments are necessary to verify whether the data presented here may constitute a model for the application of anti-oestrogen therapy for human breast cancer.

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