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Original Paper

Anti-oestrogen Stimulation of ERBB2 Ectodomain Shedding from BT-474 Human Breast Cancer Cells with *ERBB2* Gene Amplification

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Oestrogen has previously been shown to downregulate the expression of *ERBB2* oncogene in human breast cancer cells, which contain a normal non-amplified *ERBB2* gene. However, amplified *ERBB2* seems to escape from hormonal regulation. We studied shedding of the extracellular domain (ectodomain, ECD) of the *ERBB2* encoded protein in BT-474 human breast cancer cells treated with oestrogen or anti-oestrogen. Oestrogen-responsiveness of these cells has been previously demonstrated by stimulation of cell growth and expression of pS2, a marker gene known to be regulated by oestrogen receptor at transcriptional level. The concentration of the soluble ECD in the culture medium was increased by the anti-oestrogen toremifene as a function of time. In contrast, the level of *ERBB2* mRNA and protein in cell lysates was not stimulated, but was transiently suppressed by toremifene. In the presence of oestrogen, the level of ECD remained low. The increased shedding of ECD in the presence of toremifene, without parallel change in *ERBB2* transcripts (4.8 and 2.3 kb) and in cellular ERBB2 protein level, suggests that toremifene specifically contributes to the shedding of the ERBB2 ectodomain. These results show that shedding of ECD is an additional level of regulation of *ERBB2* by the anti-oestrogen toremifene. This may contribute to resistance to growth inhibition by anti-oestrogens of breast cancers which overexpress *ERBB2*.

Key words: ERBB2, *ERBB2* gene amplification, ERBB2 ectodomain, ectodomain shedding, anti-oestrogen, toremifene, breast cancer, BT-474 cells
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INTRODUCTION

THE *ERBB2/NEU* ONCOGENE product, p185, is a transmembrane glycoprotein which belongs to a family of structurally related growth factor receptors, including EGF-R, erbB3/HER-3 ([1] and the refs therein) and erbB4/HER-4 [2]. It has a cystein-rich extracellular ligand-binding domain, a single membrane-spanning domain, and a cytoplasmic domain with tyrosine kinase activity. The function and regulation of the normal *ERBB2* gene are not well understood. The gene is widely expressed in epithelial cells of human fetal and adult tissues including the mammary gland (reviewed in refs [3] and [4]). It has been reported that ERBB2 protein levels increase in rat mammary tissue during lactation [5], suggesting a function in the maintenance of the differentiated phenotype and regulation by steroid hormones.

Oncogenic activation of *ERBB2* in human tumours appears to occur through amplification and/or overexpression of the gene; no point mutations or other aberrations have been found [6]. Both amplification and overexpression of *ERBB2* have been detected at high frequency in human breast cancers, which were shown to correlate with aggressive growth of the cells and poor prognosis of the patients [7–13]. However, the mechanism responsible for the transforming potential of overexpressed *ERBB2* is still unknown [14]. Either constitutive activity of the intrinsic tyrosine kinase in the absence of the ligand, or stimulatory action of *ERBB2*-specific ligands is possible and is supported by experimental evidence [14–20].

It has been shown earlier [21] that oestrogen, a stimulator of breast cancer cell proliferation, suppresses the expression of

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ERBB2 in ZR-75-1 human breast cancer cells. These cells slightly overexpress *ERBB2*, but contain a non-amplified gene [22]. However, gene amplification seems to release *ERBB2* from oestrogen suppression [23]. This has been demonstrated in BT-474 human breast cancer cells which have approximately a 40-fold amplification of *ERBB2* gene [24] and also contain oestrogen receptors (ERs) [25–27] shown to be functional by oestrogen-stimulation of cell proliferation and pS2 mRNA expression [23]. Unlike cells which contain a normal single-copy *ERBB2* gene, in BT-474 cells the anti-oestrogen toremifene unexpectedly behaved like an oestrogen-like agonist in terms of regulation of *ERBB2* expression, demonstrated by transient downregulation of *ERBB2* mRNA and protein levels [23].

An additional mechanism which may contribute to the regulation of *ERBB2* activity and function has been revealed recently. Several groups have identified antigenic activity related to the extracellular domain (ectodomain, ECD) of ERBB2 protein in culture medium conditioned by human breast cancer cells [28–31], in sera of tumour-bearing nude mice [32] as well as in sera of breast cancer patients [33–35]. It has been suggested that the soluble ECD of ERBB2 might be involved in the extracellular competition for a cognate ligand or suppression of receptor function by heterodimer formation [31]. In the present paper, we studied the effect of the anti-oestrogen toremifene on ERBB2 expression and ECD release in human breast cancer cells which have acquired *ERBB2* amplification and are sensitive to oestrogen.

MATERIALS AND METHODS

Tissue culture

BT-474 cell line, passage #85 was obtained from ATCC (Rockville, Maryland, U.S.A.). As controls, oestrogen-sensitive MCF-7 and ZR-75-1 cell lines were used, kindly donated by Dr P. Darbre, Imperial Cancer Research Fund (London, U.K.). MCF-7 cells originated from the laboratory of Dr C.K. Osborne (University of Texas Health Science Center, U.S.A.). The cells were cultured as monolayers in plastic tissue culture dishes (Nunc, Roskilde, Denmark) in RPMI 1640 culture medium without phenol red, supplemented with 10% (BT-474 cells) or 5% (MCF-7 and ZR-75-1 cells) heat-inactivated or 2 × dextran-charcoal stripped fetal calf serum (iFCS and DC-FCS, respectively) and 2mM L-glutamine. The cells were cultured in a humidified atmosphere of 95% air, 5% CO_2, 37°C. The RPMI culture medium, FCS and L-glutamine were purchased from Gibco (Paisley, U.K.), and 17β-oestradiol (E_2) from Sigma (St Louis, Missouri, U.S.A.). E_2 and the anti-oestrogens toremifene and tamoxifen (obtained from Orion Corporation Medipolar, Oulu, Finland) were dissolved in 70% ethanol and added to the culture medium as indicated below in Figures 2–5. Cell numbers were determined as described previously [21] by counting the cell nuclei in a Coulter Counter (Coulter, Harpenden, U.K.).

For the experiments, passages #86-100 of BT-474 cells were used; 1–2.5 × 10^6 cells were plated in 5 ml of 10% DC-FCS–RPMI, without E_2 (control medium) in 50 mm diameter dishes. The next day (day 1) culture media were changed and 10 nM E_2, or 1–25 μM toremifene or 7.5 μM tamoxifen were added. The media were changed every second or third day. At the end of the experiment cells were washed and scraped in phosphate-buffered saline (PBS), and stored as a pellet in −20°C until assayed by an ERBB2 protein-ELISA. The 48-h conditioned media (total incubation time as indicated in Results) were collected from the cell cultures and centrifuged at 1000*g*, filtered (0.22 μM, Millipore, Millipore Oy, Finland) and stored at −20°C for the ERBB2 ectodomain assay.

ERBB2 ectodomain assay and immunoblotting

To analyse soluble ERBB2 protein released to the culture medium, BT-474 cells were cultured with or without E_2 or toremifene, and the conditioned media were collected from the subconfluent cultures, centrifuged and filtered (0.22 μm, Millipore) to avoid cellular contamination. The media conditioned by ZR-75-1 and MCF-7 cells were also concentrated (Centriprep 30, Amicon, Division of W.R. Grace & Co., Beverly, Massachusetts, U.S.A.). The ERBB2 ectodomain was measured with a monoclonal antibody-based solid-phase enzyme-linked immunosorbent assay (EIA, Triton Diagnostics, Alameda, California, U.S.A.) according to the manufacturer's instructions. The interassay coefficient of variation in a series of 339 breast cancer sera was 4.8% [35].

The soluble ERBB2 ectodomain from the concentrated (25-fold) BT-474 cell conditioned medium or ERBB2 protein from BT-474 cell lysates was demonstrated by immunoblotting by using a mouse monoclonal antibody against the extracellular domain of the ERBB2 protein (Cambridge Research Chemicals, U.K.). The cell pellets or lyophilised samples of concentrated conditioned medium were lysed in the electrophoresis sample buffer [2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 10% glycerol and 50 mM Tris–HCl, pH 6.8], and boiled for 5 min. The samples were centrifuged and the supernatants (containing 10 μg of protein of cell lysate or corresponding to conditioned medium from 10^7 cells) were analysed by 8% SDS–polyacrylamide gel electrophoresis (PAGE) according to Laemmli [36]. The proteins were transferred from the gels on to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Richmond, California, U.S.A.). The blotted proteins were stained by using 1:1000 dilution of the monoclonal anti-ERBB2 ectodomain primary antibody and the ECL Western blotting detection of the immunocomplexes (Amersham International plc, Amersham, U.K.). Normal mouse serum was used as a control.

ERBB2 protein assay

The cells cultured in steroid-free ("E-" control) medium were washed in PBS and centrifuged to avoid medium contamination, lysed and analysed for the non-soluble, membrane-bound cellular ERBB2 protein. The cell lysates were prepared by adding 500 μl extraction buffer (0.01 M sodium phosphate, 0.15 M sodium chloride, 1% (v/v) Triton-X 100, 0.5% sodium deoxycholate, 1% SDS and 1 mM phenylmethyl-sulphonylfluoride, pH 7.2) to the cell pellet, vortexing and incubating on ice for 15 min. The cell lysates were then centrifuged at 10 000*g* for 10 min at 4°C and the supernatant was collected. The protein concentration of the lysates was determined with the Lowry method. Samples were diluted at a concentration of 0.1 mg/ml of protein and 50 μl were used for testing in a sandwich type enzyme immunoassay (ELISA) by using a specific polyclonal antibody against the intracellular domain of the ERBB2 protein (Triton Diagnostics).

Northern blot analysis of ERBB2 *mRNA*

Total RNA was extracted from the cells in guanidine isothiocyanate by ultracentrifugation in a $CsCl_2$ gradient as described earlier [37]. Five microgrammes of total RNA were

run on denaturing 1% agarose/formaldehyde gels [38], stained with ethidium bromide (EtBr), photographed under UV-light and transferred on to GeneScreen Plus Nylon membrane (Du Pont, NEN, Boston, Massachusetts, U.S.A.) according to manufacturer's instructions. The filters were hybridised and washed as suggested by the manufacturer. The probe was a 531-bp Ava I insert of human *ERBB2* cDNA clone [39], kindly donated by Dr T. Yamamoto. The inserts were [^{32}P]-dCTP-labelled (Boehringer) by random oligonucleotide primer extension to a specific activity of approximately 10^9 cpm/μg DNA. After hybridisation and washing, the filters were exposed to autoradiographic films (X-Omat, Eastman Kodak, Rochester, New York, U.S.A. or Konica MG SR, Konica, Tokyo, Japan) at −80°C. For quantitation, the signal intensities of the autoradiographic films and the rRNAs in (the photographs of) the corresponding EtBr-stained gels were scanned by Ultroscan laser densitometer by using the GelScanXL™ software program (Pharmacia, Uppsala, Sweden). The hybridisation intensities were then corrected for rRNA of the corresponding samples [40].

RESULTS

ERBB2 ectodomain (ECD) shed into the culture medium by cells with ERBB2 *amplification*

Immunoblot analysis with a specific monoclonal antibody against the extracellular domain of the ERBB2 protein showed the presence of an immunoreactive protein of approximately 100 kD in the media conditioned by BT-474 cells (Figure 1). The concentration of this soluble protein corresponding to ERBB2 ECD was then measured in the culture media conditioned by BT-474 cells, treated with oestrogen or anti-oestrogen toremifene, by using solid-phase enzyme-linked immunosorbent assay (Triton Diagnostics). The ECD content in BT-474 cell media was higher than that of media conditioned for the corresponding time (48 h) by either ZR-75-1 or MCF-7 human breast cancer cells, in which no antigenic ECD activity could be detected (data not shown), either in the steroid deprived conditions or in the presence of toremifene.

Toremifene-increased release of ECD into the culture medium by BT-474 cells

Toremifene addition (7.5–10.0 μM, 48 h) to the BT-474 cell culture media caused approximately a 1.5–2-fold increase

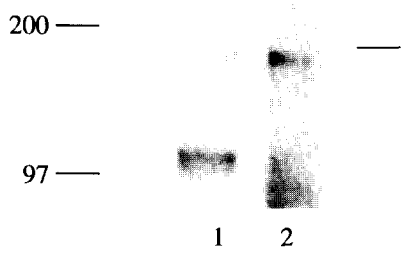


Figure 1. Immunoblot of ERBB2 ectodomain (ECD) from the medium conditioned by BT-474 cells. Lane 1, medium conditioned by 10^7 BT-474 cells, immunostained with a mouse monoclonal antibody against the extracellular part of the ERBB2 protein. Lane 2, BT-474 cell extract (10 μg of protein corresponding to approximately 2 × 10^5 cells). The position of the intact ERBB2 protein is marked on the right, and the molecular weight standards (×10^3) are shown on the left.

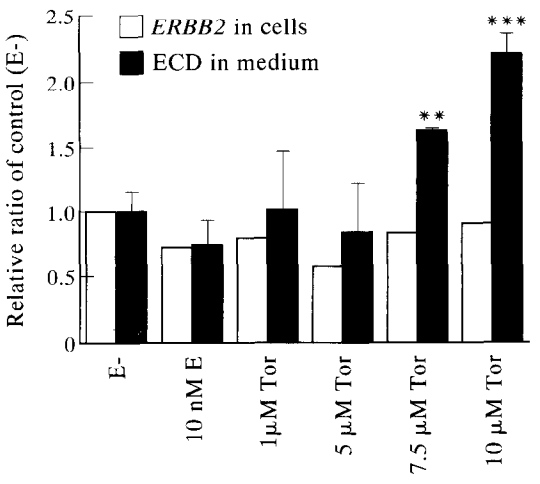


Figure 2. The expression of ERBB2 protein in BT-474 cell lysates and soluble ERBB2 ECD in the culture media after a 48-h incubation. The results are expressed as the relative ratio to the control, i.e. cellular ERBB2 content of the cells cultured in the "E-" medium and soluble ERBB2 ECD content shed to the media under the same conditions, respectively. Each bar represents the mean of two (ELISA) or three to five (± S.D., EIA) parallel cultures. All the determinations were made in duplicate. Statistical analysis: ANOVA with one between factor (group: treatment/dose), $^{**}P < 0.01$, $^{***}P < 0.001$.

in the concentration of ECD (Figure 2). In contrast, oestrogen addition (10 nM) for a corresponding time caused suppression of the ECD content in the medium. The relative change in ECD content was more pronounced after toremifene than oestrogen treatment. The highest concentration of toremifene used (25 μM, 48 h) did not cause any additional increase in the concentration of ECD (2.23-fold, when compared to the control) over that at 10 μM. The effect of tamoxifen (7.5 μM, 48 h) was similar to that of toremifene (1.82-fold when compared to the control, data not shown.) A time course study with 7.5 μM toremifene showed enhanced shedding of ECD into the culture medium as the duration of exposure increased (48–120 h), whereas an increased suppression in the presence of oestrogen was not observed (Figure 3). For comparison,

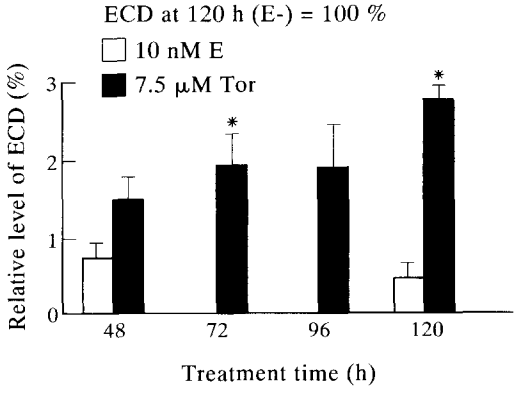


Figure 3. Time course of ERBB2 ECD concentrations in culture media conditioned by BT-474 cells with or without oestradiol (E) or 7.5 μM toremifene (Tor) for 48–120 h. The results are expressed as the relative ratio to the control, i.e. soluble ERBB2 ECD content shed to the media during the last 48 h of the 120-h culture in the "E-" medium. Two parallel culture dishes were analysed at each time point, and the experiment was repeated twice. Statistical analysis: *t*-test (paired two-sample for means, one-tail), $^{*}P < 0.05$.

the toremifene effect on ECD was also studied in an ER-negative cell line (SK-BR-3) which contain *ERBB2* gene amplification and express ERBB2 protein at a high level [24]. Toremifene did not enhance ERBB2 ECD shedding into the culture medium of these cells (values of unconcentrated media were negative by the analysis used, data not shown).

Expression of the 2.3 *kb* ERBB2 *mRNA*

In addition to the full-length 4.8 kb mRNA, the *ERBB2* gene also produces a less abundant 2.3 kb mRNA transcript, which is thought to encode the ectodomain part of the protein [31]. We detected a 2.3 kb mRNA in the Northern blots of the BT-474 cell RNA samples after prolonged exposure of the autoradiographic films (Figure 4a). An approximately 2-fold increase in the level of 2.3 kb mRNA was observed, when the levels at the beginninng (48 h) and at the end (144 h) of the BT-474 cell cultures were compared (Figure 4b). However, unlike the corresponding ECD protein shed into the culture medium, the level of the 2.3 kb mRNA increased irrespective of the hormones administered, i.e. both in the presence of toremifene and oestrogen.

Weak anti-oestrogen effect on BT-474 cell growth

Oestradiol (10 nM) enhanced the growth rate of BT-474 cells approximately 1.5–2.5-fold, when compared to the growth rate of cells cultured without oestrogen (i.e. in the medium with 10% dextran-charcoal-stripped FCS and no oestrogen addition, "E-") (Figure 5a,b). In spite of the clear growth stimulation by oestrogen, the anti-oestrogen had less effect. At a 1.0 μmol/l concentration toremifene had a small agonistic effect, i.e. it stimulated cell growth to some extent compared to the control ("E-") (Figure 5a). At a concentration of 7.5 μmol/l, toremifene had an inhibitory effect on the net cell number when compared to control ("E-"), but the cells were still able to grow (Figure 5b). The effect of toremifene was reversible, since withdrawal of toremifene and addition of oestrogen (E+ rescue) to the culture medium was able to enhance the cell proliferation (Figure 5b). Toremifene only slightly opposed the growth stimulation by oestrogen, when oestrogen was added together with toremifene (Figure 5b, Tor 7.5 μM→E + Tor).

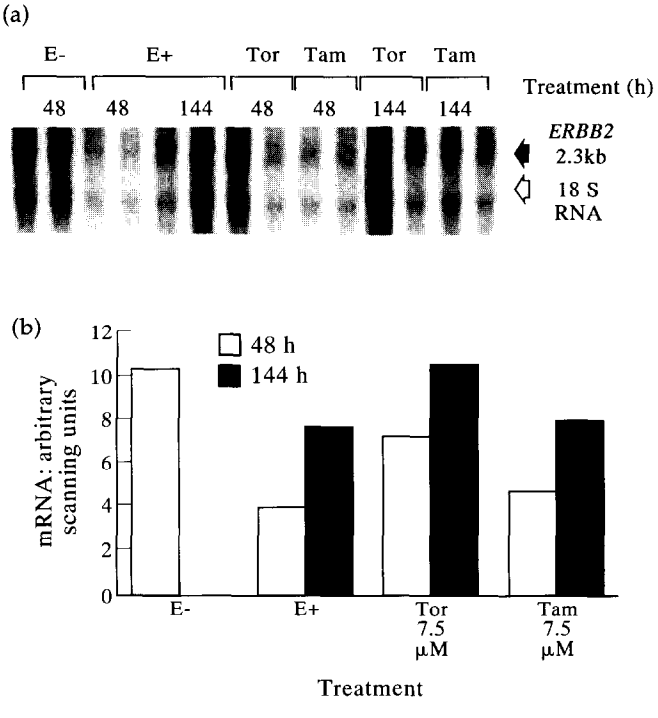


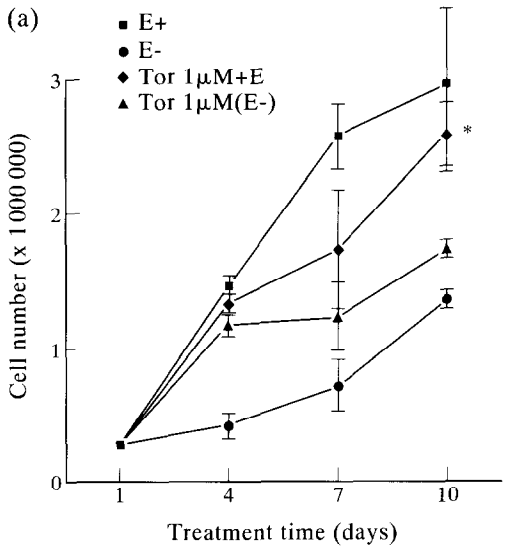
Figure 4. A Northern blot (a) and quantitation (b) of the 2.3 kb *ERBB2* mRNA levels in BT-474 cells after 48 or 144 h culture without (E-) or with 10 nM oestradiol (E+) or 7.5 μM toremifene (TOR) or tamoxifen (TAM). A Northern blot of total RNA (5 μg/lane). After hybridisation of the filters with *ERBB2* AvaI probe [39], the autoradiographic films were exposed for approximately 40 times longer, compared to the 4.8 kb mRNA. 18S rRNA (2.0 kb) was used as a size marker. For quantitation of the *ERBB2* mRNA levels in the Northern blot, the signal intensities were scanned and corrected for RNA loading, as described in the Materials and Methods. Each RNA sample was prepared from two to three parallel dishes. The cultures and Northern blot analysis were repeated twice.

DISCUSSION

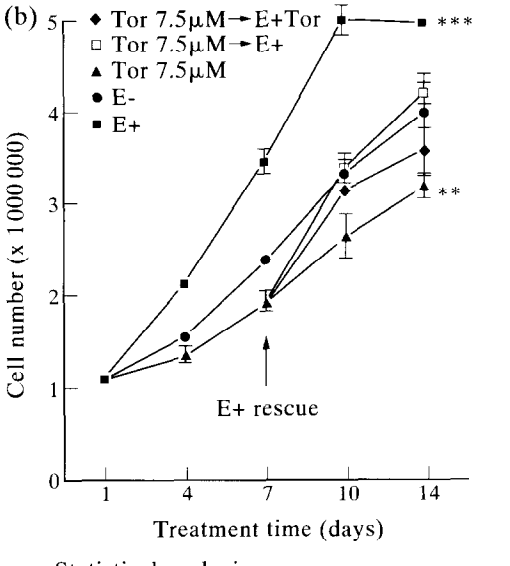
In the present study, we demonstrated that the extracellular domain (ectodomain, ECD) of ERBB2 protein is shed into the culture medium by human breast cancer cells, which contain an amplified *ERBB2* gene. Moreover, the shedding of ECD increased in the presence of the anti-oestrogen toremifene as a function of time. This was accompanied by cell growth, which was only slightly inhibited by a high concentration of toremifene but stimulated by oestrogen.

The soluble ECD content of media conditioned by BT-474 cells was high and corresponded to the high concentration of the membrane-bound ERBB2 protein in BT-474 cell lysates. This cellular ERBB2 protein content was high when compared to media conditioned by ZR-75-1 and MCF-7 cells, in which ERBB2 protein was undetectable by this method. The shed ECD showed a molecular weight of approximately 100 kD in SDS-PAGE. This result is in accordance with those reporting soluble ECD in the serum samples of patients with *ERBB2* overexpressing breast tumours [33–35], in the sera of tumour-bearing nude mice [32] and in cell culture media conditioned by human breast cancer cells [28–31], i.e. a molecular weight within a range of 100–130 kD. (Multiple sizes have been reported even in the same sample [29].) This is close to the predicted size (118 kD) of the putative extracellular part of the unfragmented 185 kD ERBB2 protein. In addition, the levels of ECD paralleled the expression levels of cellular ERBB2 protein. A new finding in our study was that the ECD concentrations were significantly ($P < 0.01$) stimulated by toremifene, although the expression of mRNA and cellular ERBB2 protein remained unaffected. The effect of toremifene on the ECD content also exceeded the effect of oestrogen-withdrawal in BT-474 cells, and was not observed in ER-negative cells containing *ERBB2* gene amplification. These data indicate that toremifene specifically contributes to the production of the ERBB2 ectodomain.

The mechanism of synthesis and/or shedding of soluble ECD is unclear. Gene rearrangement, alternative splicing and proteolytic cleavage are all possible and have been shown for other membrane receptors such as CSF-R, IL2-R and EGF-R (refs in [29]) and ERBB3 [41]. Recently, Scott and coworkers [31] reported that a truncated ERBB2 protein was encoded by a 2.3 kb mRNA produced by alternative splicing of *ERBB2* gene transcript in BT-474 and SK-BR-3 cells. This was reported to correspond to the ectodomain protein of approximately 100 kD, and both secreted and intracellular forms were found [31]. The complex 4.8 kb mRNA was approximately 40 times more abundant than the 2.3 kb mRNA in all six breast cancer cell lines (including BT-474) studied [31]. We



Statistical analysis:
Tor 1μM+E versus E+ curve, $*P<0.05$
Tor 1μM (E-) versus E- curve, n.s.



Statistical analysis:
E+ versus control E- curve, $***P<0.001$
Tor 7.5 mM versus control E- curve, $**P<0.01$

Figure 5. The effect of oestrogen and anti-oestrogen toremifene on BT-474 cell growth. The cells were plated in 5 ml (a) or 3 ml (b) of phenol red-free RPMI 1640 with 10% dextran-charcoal treated FCS, without oestradiol ("E-" control medium) in 50 mm (a) or 30 mm (b) diameter dishes. The next day (day 1) culture media were changed and 10 nM oestradiol ("E+"), or 1.0 μM toremifene (with or without 10 nM oestradiol) or 7.5 μM toremifene (TOR) were added. The cell numbers were determined by counting the cell nuclei in a Coulter Counter (Coulter, Harpenden, U.K., [21]) in triplicate from five (a) or three (b) parallel dishes (mean ± S.D.). The figure shows representative curves from several similar experiments. Statistical analysis: ANOVA with two between factors (time, treatment), from day 4 to day 10 or 14. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

also detected a 2.3 mRNA in Northern blots after a long exposure time. Approximately 2-fold increase in the mRNA levels was detected during anti-oestrogen treatments as a function of time. However, the 2.3 mRNA level was also doubled in the presence of oestrogen, although the ECD content in the medium simultaneously decreased. This indicates that the toremifene-induced increase of ECD cannot be explained by increased production of the 2.3 kb transcript alone. Thus, it is likely that post-transcriptional mechanisms, such as proteolytic cleavage of cell surface protein, are involved in the prodcution of the ERBB2 ectodomain during anti-oestrogen treatments. The results show that shedding of ECD is an additional level of regulation of *ERBB2* by anti-oestrogens. The shed ECD might trap the ligand of the ERBB2 receptor protein and/or interefere with receptor function (dominant negative receptor dimers), subsequently affecting the ERBB2 mediated signal transduction pathway leading to insensitivity to growth regulatory factors [27, 31].

Regulation of *ERBB2* mRNA and protein expression seems to be different in cells which contain a normal, single-copy *ERBB2* gene [21] and in cells that have acquired *ERBB2* amplification. We have shown previously in ZR-75-1 human breast cancer cells, which modestly overexpress *ERBB2*, but contain a normal single-copy gene (mRNA expression level about 8-fold compared to that of HBL-100 cells [22]) that *ERBB2* mRNA expression was downregulated by oestrogen and induced by the anti-oestrogen toremifene [21]. Corresponding results have also been obtained with other breast cancer cells (T47D and MCF-7 cells) [5, 42–44] which have a normal *ERBB2* gene copy number. Thus, oestrogen down-regulation of the *ERBB2* gene seems to be a common finding in breast cancer cells with no gene amplification, and it suggests that the *ERBB2* gene is under oestrogen control in normal human mammary epithelium. Observations from the lactating rat mammary gland support this hypothesis [5] and further suggest that *ERBB2* expression in normal (mammary) cells may be associated with a differentiated state rather than with high growth rate. In BT-474 cells, which in addition to ERs [25, 26], shown to be functional by stimulated cell proliferation and pS2 expression [23]), also contain a 40-fold *ERBB2* amplification [24], the suppression of *ERBB2* mRNA and protein by oestrogen was transiently similar to that of a single-copy gene, i.e. it resulted initially in a 4-fold difference in mRNA levels and less than 1.5-fold difference in protein levels depending on the presence of oestrogen in the culture media. This suppression by oestrogen diminished after prolonged culture, and finally the expression of both mRNA and protein became independent of the presence of oestrogen [23]. Strikingly, the anti-oestrogen toremifene acted as an oestrogen-like agonist in terms of its effect on *ERBB2* expression, transiently downregulating *ERBB2* mRNA and protein levels [23].

Clinically, most ER-positive *ERBB2* amplified tumours are resistant to tamoxifen treatment, which correlates with poor clinical outcome of breast cancer patients [45, 46]. A corresponding result was also found in MCF-7 cells, which became tamoxifen resistant after transfection of *ERBB2*, but whose growth was still dependent on oestrogen [26]. In our study, the growth of BT-474 cells was similarly sensitive to oestrogen, but the effect of toremifene was less antagonistic as it is in most "truly" oestrogen-sensitive cell lines [21, 47]. Moreover, in the oestrogen rescue experiment, toremifene could only slightly oppose oestrogen effect even at the concen-

tration 7.5 μmol/l. In conclusion, *ERBB2* gene amplification may lead to cell growth which cannot be suppressed by anti-oestrogens despite the fact that it was induced by oestrogen. The stimulated shedding of ECD may be a crucial factor contributing to or causing resistance to growth inhibition by anti-oestrogens.

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