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Effects of the Microtubule-disturbing Agents Docetaxel (Taxotere®), Vinblastine and Vincristine on Epidermal Growth Factor-receptor Binding of Human Breast Cancer Cell Lines *In vitro*

A.-R. Hanauske, H. Depenbrock, D. Shirvani and J. Rastetter

Epidermal growth factor (EGF) is a mitogenic peptide that binds to surface membrane receptors (EGFR) of breast cancer cells. After binding, secondary transmitter molecules are activated by tyrosine phosphorylation of the intracellular receptor domaine. The activity of the EGF/EGFR system can be modulated by a variety of chemically unrelated compounds including cytostatic agents. The purpose of our present study was to determine the effects of mitotic inhibitors on EGF receptor binding on human breast cancer cells. We found that MDA-231 and MDA-468 cells bind substantially more [^{125}I]EGF after preincubation with docetaxel, vinblastine and vincristine. This effect was concentration- and time-dependent, reaching a maximum at 3000 ng/ml and 48 h incubation for docetaxel, and 100 ng/ml and 48 h incubation for vinca alcaloids. Subsequent experiments showed an increase in the rate of EGF binding as well as maximal binding capacity. Scatchard analysis of binding experiments under equilibrium conditions indicated that this was due to an increase in the number of apparent EGF binding sites. Modulation of EGF receptor binding by docetaxel, vinblastine, and vincristine was not detectable when isolated membranes were used, indicating that intact cytoplasmatic mechanisms are required for the upregulation of EGF receptors.

Key words: epidermal growth factor, receptor binding, docetaxel, vinblastine, vincristine, breast cancer
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INTRODUCTION

EPIDERMAL GROWTH factor (EGF) is a mitogenic polypeptide with a molecular mass of 6200 D. Following binding of EGF to a specific cell surface receptor, the ligand/receptor complex is internalised. Evidence exists that some EGF receptors may recycle to the cell surface [1, 2], although eventually the receptors are degraded. Exposure to EGF leads to downregulation of its receptor [3]. There is convincing evidence that growth factor receptors may be modulated not only by their physiological ligand but also by other agents. These include other growth factors, interferons, tumour necrosis factor, hormones and low molecular weight compounds like phorbol esters. In addition, antineoplastic drugs have been reported to modulate EGF binding to its surface receptor [4], and there is evidence that doxorubicin may upregulate the EGF receptor on fibroblasts [5]. In earlier studies, we reported a loss of EGF receptors on breast cancer cells following incubation with pirarubicin [6].

Mitotic inhibitors belong to the most active group of agents in cancer chemotherapy, and their major mechanism of action is their binding to the mitotic spindle which leads to growth arrest of premitotic cells. In addition, several lines of evidence suggest that vinca alkaloids may modulate tumour growth factors or their membrane surface receptors. First, vinblastine down-regulates the number of insulin receptors on isolated muscle cells from adult rat heart [7]. Second, rodent tumour cell lines and human neuroblastoma cell lines, selected for high levels of resistance to vincristine, express increased numbers of EGF membrane receptors [8, 9]. The purpose of our study was to determine the effects of docetaxel, vinblastine, and vincristine on EGF receptor binding to human breast cancer cells *in vitro*, and to determine whether any drug-mediated effect may be due to a direct action on cell membranes.

MATERIALS AND METHODS

MDA-231, and MDA-468 human breast cancer cells were kindly provided by Dr C. K. Osborne (University of Texas, San Antonio, Texas, U.S.A.). Clinical preparations of vinblastine and vincristine were obtained from Lilly Deutschland GmbH (Bad Homburg, Germany). Docetaxel was kindly provided by Rhône-Poulenc Rorer S.A. (Vitry-sur-Seine, France). Recombinant human EGF was purchased from Flow Laboratories

Correspondence to A.-R. Hanauske.
The authors are at the Division of Haematology and Oncology, I. Medical Department, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, D-81675 München, Germany.
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(Meckenheim, Germany). Recombinant human [^{125}I]EGF was obtained from Amersham Buchler (Braunschweig, Germany) at a specific activity of 47–52 TBq/mmol. MDA-231 cells were cultured in Richter's improved MEM Zinc Option Medium containing 10% fetal calf serum (Gibco). MDA-468 cells were cultured in MEM alpha medium with 10% fetal calf serum (Gibco). All dilutions of docetaxel were prepared in distilled water to give the final concentrations of 3.2–3200 ng/ml (0.004–4 μM). All dilutions of vinblastine and vincristine were prepared in distilled water to give final concentrations of 0.1–1000 ng/ml. One hundred thousand cells per well were seeded in 12-well plates (Tecnomara, Fernwald, Germany), and incubated at 37°C, 5% CO_2, 100% humidity. When subconfluent, the drugs were added at the specified concentrations and incubated for 0.5–48 h. EGF receptor binding studies and Scatchard analyses were performed as described earlier [4, 10]. Cell membranes were prepared using the nitrogen cavitation method in the presence of 50 μg/ml leupeptine (Fluka, Neu-Ulm, Germany) [11]. The crude homogenate was centrifuged at 12 000 *g* for 20 min and the supernatant was centrifuged a second time at 100 000 *g* for 1 h. The cell membrane fraction was then resuspended and repeatedly aspirated through a 26G needle. [^{125}I]EGF binding experiments were performed at 37°C after preincubation of membrane aliquots corresponding to 4–6 × 10^6 cells with various concentrations of the drugs. For determination of the cytotoxic effects of the drugs, 10^5 cells were seeded into 25 cm^2 tissue culture flasks (Tecnomara). When subconfluent, the drugs were added for 24–48 h. At the end of the incubation periods, cells were washed, collected, and counted. Viability was determined using trypan blue dye exclusion. An aliquot of the cell suspension was used for determination of soft agar cloning efficiency. For cell cloning experiments, untreated and treated cells were seeded into a mixture of 0.3% agar (Sigma) in their respective culture media over a base-layer of 0.5% agar. Each experiment contained a "positive" control for cell kill to detect possible contamination by cell clumps [12]. After 14 days, colonies (≥50 μm) were counted by use of an inverted microscope. Cloning efficiencies were calculated using the formula: number of colonies counted × 100/number of cells seeded = cloning efficiency (%).

RESULTS

Figure 1a–f summarise concentration-dependent effects of docetaxel, vinblastine, and vincristine on [^{125}I]EGF receptor binding by MDA-231 and MDA-468 breast cancer cells. The cells were not affected at concentrations less than 10 ng/ml. After exposure to higher concentrations of the agents, a significant increase in EGF binding was observed in both cell lines. In addition, modulation of [^{125}I]EGF receptor binding by the drugs was also time-dependent with a maximum modulation occurring beyond 15 h exposure. In MDA-231 cells, binding of [^{125}I]EGF was increased by docetaxel, vinblastine and vincristine up to 2.02 ± 0.02, 2.60 ± 0.47 and 2.39 ± 0.16 fold respectively. In MDA-468 cells, binding of [^{125}I]-EGF was increased by docetaxel, vinblastine and vincristine to 2.08 ± 0.06, 6.06 ± 2.26 and 3.69 ± 2.65 fold, respectively. In both cell lines, maximal EGF receptor modulation was consistently observed after preincubation with 3200 ng/ml of docetaxel for 48 h. In MDA-231 cells, maximal EGF receptor modulation was observed after preincubation with 1000 ng/ml vinblastine or vincristine for 48 h. In MDA-468 cells, maximal EGF receptor modulation was observed after preincubation with 100 ng/ml vinblastine or vincristine for 48 h.

The kinetics of [^{125}I]EGF binding following preincubation with the drugs was determined by using subconfluent monolayers of MDA-231 and MDA-468 cells exposed to docetaxel, vinblastine and vincristine at final concentrations of 320 or 3200 ng/ml (docetaxel) and 10 or 100 ng/ml (vinblastine and vincristine) for 24 or 48 h. After removal of the drug, 1.6 × 10^{-11} M [^{125}I]EGF was added in 20 nM HEPES. Binding was determined at various time points between 0.5 and 24 h at 0°C. As shown in Figure 2a–f, binding of [^{125}I]EGF to its receptor was substantially modulated by preincubation with docetaxel, vinblastine and vincristine. In MDA-231 cells, at 24 h, EGF binding was increased 2.8-fold with preincubation with doxetaxel (3200 ng/ml), 3.0-fold with vinblastine (100 ng/ml), and 2.1 fold with vincristine (100 ng/ml). In MDA-468 cells, at 24 h, EGF binding was increased 2.1-fold, with preincubation with doxetaxel (3200 ng/m) 2.5-fold with vinblastine (100 ng/ml) and 2.3-fold with vincristine (100 ng/ml).

Subsequent experiments were performed to further characterise the growth factor modulating effects of the drugs. MDA-231 and MDA-468 cells were exposed to docetaxel (320 ng/ml), vinblastine (100 ng/ml) or vincristine (100 ng/ml) for 24 h at 37°C. After removal of the drug, EGF receptor binding was studied in a competitive assay at 0°C to avoid receptor internalisation. Scatchard analyses of binding data under equilibrium conditions are shown in Figure 3a and b. In MDA-468 cells, incubation with docetaxel did not alter receptor affinity. In control experiments, a K_d of 0.49 × 10^{-9} M was observed as compared with a K_d of 0.47 × 10^{-9} M with docetaxel. The number of apparent EGF binding sites, however, increased from 2 088 000/cell to 3 670 000/cell. For MDA-231 cells, a moderate decrease in receptor affinity was observed with the K_d changing from 1.2 × 10^{-9} M to 2.0 × 10^{-9} M. Again, the number of apparent binding sites was increased from 90 000/cell to 167 000/cell. Similar data were obtained for the other drugs (data not shown). These data suggest that the observed increase in [^{125}I]-EGF binding is mainly due to an increase in binding sites rather than to alterations in receptor affinity.

In order to determine whether the modulation of EGF receptor binding by the drugs is due to a direct effect on the cell membrane, binding experiments were performed using isolated membranes from MDA-231 and MDA-468 cells. Neither docetaxel nor vinblastine or vincristine altered binding of [^{125}I]EGF to cell membranes after 24 and 48 h of preincubation (data not shown). These results indicate that the observed modulation of EGF binding by these agents was not the result of a direct effect on cell membranes. It rather represents an event that requires intact cytoplasmatic mechanisms. Tables 1–3 summarise the effects of docetaxel, vinblastine and vincristine on cell number, viability, and cloning efficiency. After incubation with the highest concentrations, the large majority of cells remained viable.

DISCUSSION

Growth of normal cells is controlled, in part, by the interaction of growth factors with cellular receptors. Malignant transformation has long been associated with the deregulation of growth factor and growth factor receptor expression. Binding of EGF to its receptor (EGFR) activates an intrinsic receptor tyrosine kinase, and initiates a signal transduction pathway resulting in ligand-induced responses in the target cell. Constitutional overexpression of the EGFR gene or gene product has been reported for numerous human cancers of epithelial origin including breast, gastric, lung and renal carcinoma and have been

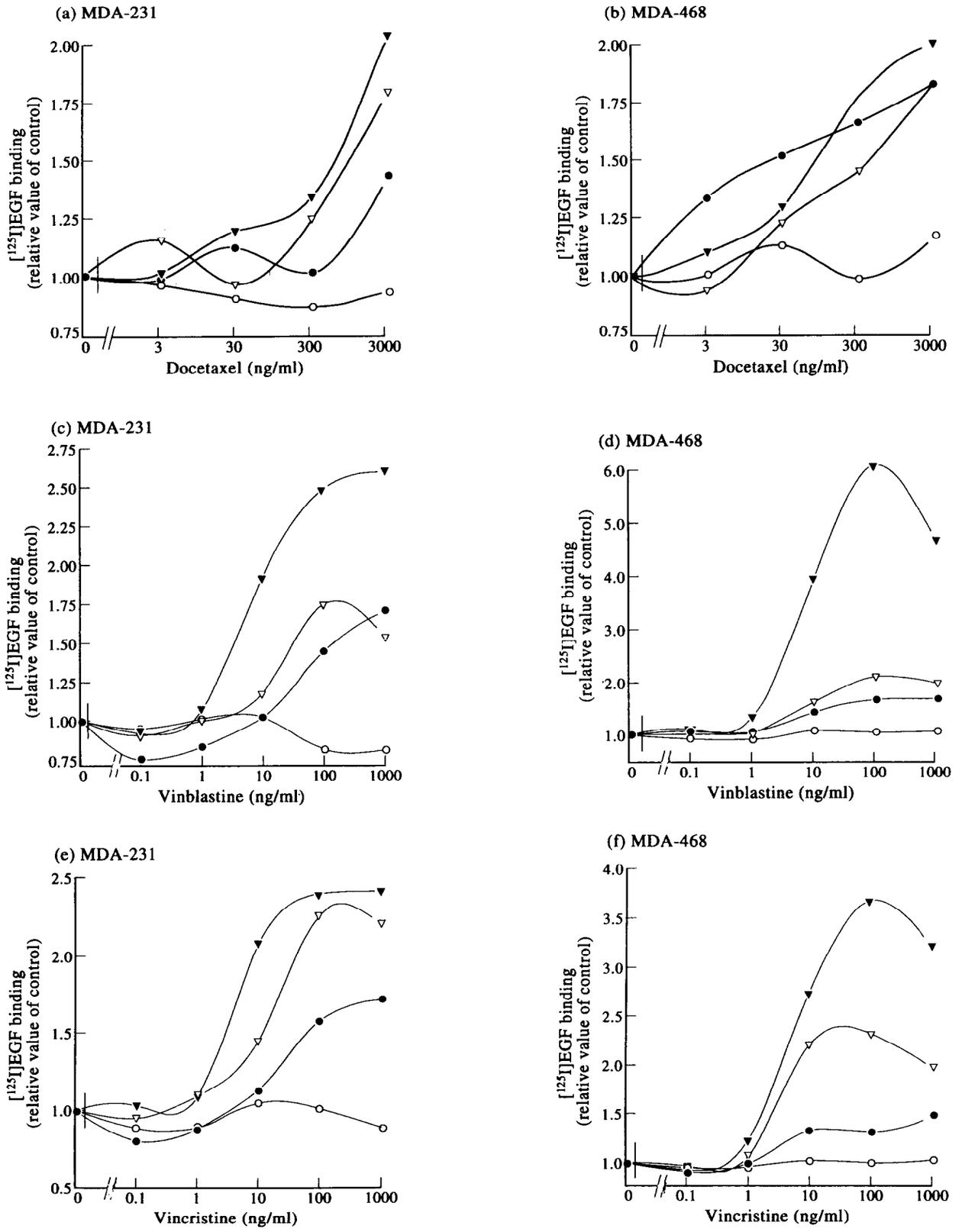

Figure 1. EGF receptor binding to breast cancer cells after incubation with docetaxel, vinblastine and vincristine. EGF receptor binding was performed as described in [10]. Data are expressed as a relative value of the control (test control) and are means of at least two separate experiments. The standard error of the mean of the original experiments was ≤0.01. Docetaxel modulated EGF receptor binding in a concentration- and time-dependent manner with maximal effects at 3200 ng/ml and 48 h. Vinblastine and vincristine modulated EGF receptor binding in a concentration- and time-dependent manner with maximal effects at 100 ng/ml to 1000 ng/ml and 48 h. Preincubation: ○ 0.5 h; ● 15 h; ▽ 24 h, ▼ 48 h.

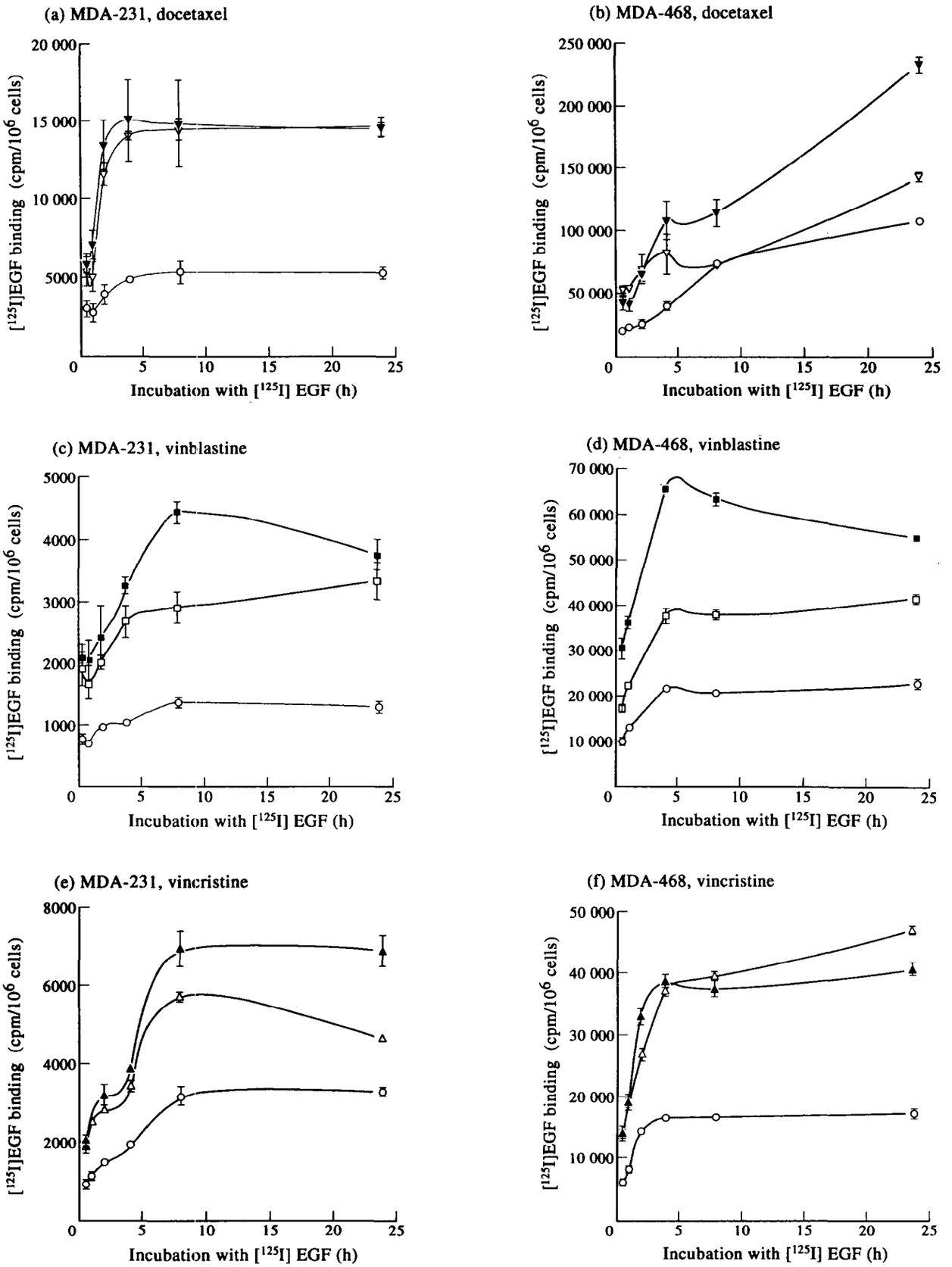

Figure 2. Kinetics of [^{125}I]EGF receptor binding to breast cancer cells. Data are means of triplicate determinations. Docetaxel, vinblastine, and vincristine substantially increased EGF binding to MDA-231 and MDA-468 cells. ○ Control; ▽ docetaxel (320 ng/ml); ▼ docetaxel (3200 ng/ml); □ vinblastine (10 ng/ml); ■ vinblastine (100 ng/ml); △ vincristine (10 ng/ml); ▲ vincristine (100 ng/ml).

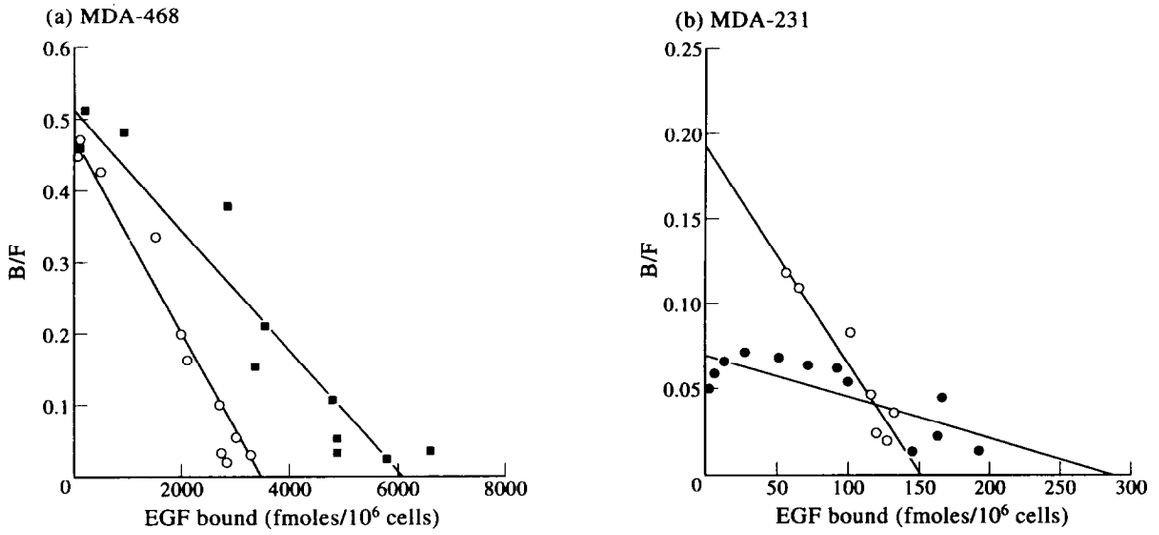

Figure 3. Scatchard analysis of competitive binding experiments under equilibrium conditions. Binding conditions were 4 h (MDA-468) or 8 h (MDA-231), 0°C, to prevent receptor internalisation. Docetaxel increased the number of apparent binding sites for EGF with no effects on binding affinity. Vinblastine increased the number of apparent binding sites for EGF with moderate effects on binding affinity. ○ Control; ■ docetaxel; ● vinblastine.

correlated with poor patient prognosis. These findings suggest that magnification of EGFR-mediated cellular events may be related to the transformed phenotype of epitheloid cells [13–16]. In addition to gene amplification, several compounds are able to functionally interfere with the EGF/EGFR system. These agents include transforming growth factor-α, transforming growth factors-β, platelet-derived growth factor, tumour necrosis factor, nerve growth factor, bombesin, phorbol esters, vasopressin, interferons, as well as several non-vinca neoplastic drugs [5, 17–23]. However, even for single compounds, it is presently difficult to derive a predictive model for tumour growth factor interactions. For interferons, for example, both up- and down-regulation of EGF receptors have been reported depending on the experimental system used [17–19].

In the present study we have found that the microtubule-polymerising agent, docetaxel, as well as the microtubule-disrupting agents, vincristine and vinblastine, alter receptor binding of EGF to human breast cancer cells. This effect was observed in both MDA-231 and MDA-468 cell lines. Microtubules are part of the cytoskeleton, and appear to play a major role in the function and processing of a variety of cell membrane receptors in non-malignant and malignant cells [24–32]. While few reports have been published on the role of microtubules for the EGF receptor system, most investigators

Table 1. Effects of docetaxel (3200 ng/ml) on cell number, viability, and soft agar clonogenicity of breast cancer cell lines. Docetaxel decreased cell number and clonogenicity. Viability as determined by trypan blue exclusion was ≥80%

	Number of cells/ flask ($\times 10^4$)	Viability (%)	Cloning efficiency (%)
24 h			
MDA-231			
Control	25.0	93	5.3
Docetaxel	13.0	96	1.1
MDA-468			
Control	8.0	94	1.9
Docetaxel	1.0	100	1.1
48 h			
MDA-231			
Control	20.5	95	4.8
Docetaxel	6.5	92	1.1
MDA-468			
Control	7.5	93	2.8
Docetaxel	1.3	80	1.2

Table 2. Effects of vinblastine (100 ng/ml) on cell number, viability, and soft agar clonogenicity of breast cancer cell lines. Vinblastine decreased cell number and clonogenicity. Viability as determined by trypan blue exclusion was ≥86%

	Number of cells/ flask ($\times 10^4$)	Viability (%)	Cloning efficiency (%)
24 h			
MDA-231			
Control	20.5	96	6.3
Vinblastine	8.8	89	1.9
MDA-468			
Control	10.0	100	15.9
Vinblastine	5.3	86	1.8
48 h			
MDA-231			
Control	18.0	93	7.0
Vinblastine	4.0	94	1.4
MDA-468			
Control	15.8	100	6.9
Vinblastine	3.5	93	1.6

Table 3. Effects of vincristine (100 ng/ml) on cell number, viability, and soft agar clonogenicity of breast cancer cell lines. Vincristine decreased cell number and clonogenicity. Viability as determined by trypan blue exclusion was ≥80%.

	Number of cells/ flask ($\times 10^4$)	Viability (%)	Cloning efficiency (%)
24 h			
MDA-231			
Control	20.5	96	6.3
Vincristine	6.3	80	1.3
MDA-468			
Control	10.0	100	15.9
Vincristine	6.3	100	2.2
48 h			
MDA-231			
Control	18.0	93	7.0
Vincristine	3.3	92	1.4
MDA-468			
Control	15.8	100	6.9
Vincristine	4.3	100	1.9

have focused on transferrin receptors. Recently, Jin and Snider reported on an increase in the number of surface transferrin receptors after incubation of K562 cells with nocodazole [33]. This finding supports our observation of increased numbers of EGF receptors, which was due to altered receptor dynamics. We have not determined the rate of EGF receptor endocytosis, but receptor endocytosis was found to be substantially depressed in the transferrin system after exposure to nocodazole. Jin and Snider also reported an increase in receptor recycling through the Golgi complex [33], a pathway that may also be of potential interest for EGF.

The functional status of growth factor receptors after incubation with spindle-active agents is unclear. However, it may be speculated that these receptors lack full biological competence since intact microtubuli are apparently required for signal transduction after activation of mitogen-activated protein (MAP) kinases and MAP kinase kinases [28, 34–36].

In summary, our results demonstrate that spindle-active agents like docetaxel, vinblastine and vincristine increase growth factor receptor binding to breast cancer cells due to a mechanism that requires cytoplasmatic components. This indicates that microtubules appear to play a role for EGF receptor processing and subsequent signal transduction.

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Relationships Between Tamoxifen Binding Proteins in Primary Breast Cancer Biopsies

S. Marsigliante, G. Leo, M. D'Elia, G.P. Vinson, S. Greco, J. Puddefoot and C. Storelli

Using high-resolution isoelectric focusing gel electrophoresis (IEF), two tamoxifen binding sites (TBS) with isoelectric point (pI) values of 4.5 and 4.3 were identified, with different affinities for tamoxifen. The form at pI 4.3 (HTBS) displayed high affinity for the ligand (kD ≈ 5 nM), while the protein at pI 4.5 (LTBS) had lower affinity (kD ≈ 50 nM). LTBS was found in the microsomal fraction and HTBS in the cytosol. Of a total of 319 tumours studied, 257 were oestrogen receptor (ER) positive and 106 HTBS positive. In this combined group, thus able to bind tamoxifen either through the presence of ER or HTBS (or both), ER and PR were both negatively correlated with HTBS ($P < 0.0001$). The oestrogen-induced protein pS2 was assayed in 92 of the 319 tumours, and was also negatively ($P < 0.0001$) correlated with HTBS. The levels of HTBS were similar between infiltrating ductal carcinomas without special features (NOS) and non-NOS forms. However, HTBS concentrations were significantly higher in poorly differentiated grade 3 carcinomas than grade 2 ($P < 0.05$) and grade 1 ($P < 0.01$) forms. Conversely, ER concentration was lower in grade 3 than grade 1 forms ($P < 0.05$). Both the relationship between high affinity TBS and ER and the high concentration of HTBS in ER-poor grade 3 carcinomas may have a bearing on the known variability of tumour response to endocrine therapy and prognosis.

Key words: breast cancer, oestrogen receptor, tamoxifen binding sites, antioestrogen binding sites, progesterone receptor, pS2

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INTRODUCTION

THE TRIPHENYLETHYLENE, tamoxifen, shows growth inhibitory effects on oestradiol target tissues [1] and cell lines [2, 3] by competitive binding to the oestrogen receptor (ER). In breast cancer, significantly improved survival rates and prognosis follow tamoxifen treatment [4]. These effects are maximal if the excised primary tumour is ER positive, but tamoxifen may exert its growth suppressive effects and also reduce controlateral and vascular disease even in patients with ER-negative tumours [5, 6]. This suggests that the hormonal action of tamoxifen may be mediated through another mechanism in addition to binding to ER. One possibility is that specific tamoxifen binding sites (TBS), which have been demonstrated both in normal breast and in tumours [7, 8], may be involved. Such binding sites have a broad intracellular localisation, in that they have been identified both in the microsomal or low speed