Granulocyte-colony stimulating factor upregulates ErbB2 expression on breast cancer cell lines and converts primary resistance to trastuzumab

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The recombinant monoclonal antibody trastuzumab has antiproliferative effect on breast cancer (BC) cells with ErbB2 overexpression. We postulated that a mechanism able to modify ErbB2 expression enhances the antitumor effect of trastuzumab. We analyzed whether granulocyte-colony stimulating factor (G-CSF), widely used in adjuvant cancer therapy to alleviate chemotherapy-induced myelotoxicity, could influence ErbB2 expression in BC cells and patients. The expression of ErbB2 (Herceptest) was analyzed in four BC cell lines (BT474, SKBR3, ZR75.1, and T47D) treated with G-CSF and in five samples biopsies from BC patients subjected to G-CSF rescue after chemotherapy. The effects of G-CSF and trastuzumab alone or their combination on cell growth and apoptosis were investigated. G-CSF receptor was detected on all cell lines and BC patients. G-CSF induced upregulation of ErbB2 in SKBR3, ZR75, and T47D cells. This modulation was not associated with an increase in tumor cell growth *in vitro*. Trastuzumab alone inhibited colony formation in soft agar but did not induce apoptosis on BC cells with no or low ErbB2 genomic amplification. The combination of trastuzumab and G-CSF enhanced the inhibition of tumor colony formation and induced apoptosis on these cells. This effect was further increased by G-CSF pretreatment. Five of nine BC patients showed an increase of Herceptest score after G-CSF administration. G-CSF treatment increases ErbB2 expression *in vitro* and *in vivo* enhancing the activity of trastuzumab on BC cell lines inducing apoptosis of BC cells with low or no ErbB2 genomic amplification. *Anti-Cancer Drugs* 19:689–696 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The ErbB2/HER2/neu proto-oncogene encodes a tyrosine kinase receptor [1,2], overexpressed in 20–30% of human breast cancers (BCs) [3–7]. The oncogenic potential of ErbB2, its high level of expression, and cell surface localization in BC make this protein a target for antitumor therapeutic approaches. Detection of ErbB2 is useful for the selection of BC patients suitable for treatment with trastuzumab [8,9], a recombinant monoclonal antibody against an extracellular domain of ErbB2. Trastuzumab has a marked antiproliferative effect and increases the effect of chemotherapy in BC that overexpresses ErbB2 [10–18]. The activity of trastuzumab is specific for malignant cell lines or xenograft with ErbB2 overexpression and has no effect on cells with physiological levels of the receptor [19,20].

Several methods have been used to assess HER2 status in BC patients. The Food and Drug administration approved the Herceptest [21–23], an immunohistochemistry procedure, to monitor ErbB2 protein expression in breast cancer, and fluorescence in-situ hybridization [24] to determine HER2 gene copy number. The benefit of trastuzumab is now proven for patients with Herceptest score 3+ or HER2 gene amplification [25]. Factors that modify the ErbB2 protein expression may theoretically change the responsiveness of BC cells to antibody-based therapy.

Recently, de Alava and coworkers [26] demonstrated that transmembrane neuregulin expression is frequent in BC patients and its expression correlate with the response to trastuzumab-based therapy. Patients without ErbB2 amplification, but with high levels of neuregulin expression, respond to treatment. These results suggest that other parameters modulating ErbB2 expression or activation in the absence of ErbB2 genomic amplification might be considered to enlarge the spectrum of BC patients who may benefit from trastuzumab.

We explored the hypothesis that some drugs are able to modify ErbB2 expression on BC cells. As the most common grade 3/4 toxicity in patients receiving

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a combination of trastuzumab and docetaxel is neutropenia [27], a considerable fraction of them might need myelopoietic stimulation by growth factors. For this reason we considered the effect of granulocyte-colony stimulating factor (G-CSF) on ErbB2 expression in BC cell lines and in BC patients. Interestingly, we demonstrated that G-CSF increases both in-vivo and in-vitro ErbB2 protein level without affecting BC cell proliferation in the in-vitro model. Furthermore, G-CSF treatment could induce susceptibility to trastuzumab on ErbB2$^+$ BC cells with no or low genomic amplification, which are generally not responsive.

Materials and methods

Tumor samples, cell lines, and treatments

The BC cell lines SKBR3, T47D, ZR75.1, MCF7, and BT474, were maintained in Dulbecco Modified Eagle Medium (DMEM) (Invitrogen, San Giuliano, Milan, Italy) with 10% fetal bovine serum (FBS; Celbio, Milan, Italy). The MCF10A human mammary epithelial immortalized cell line was cultured in 1 : 1 DMEM/Ham-F12 plus 10 μg/ml insulin, 5 μg/ml transferrin, 0.5 μg/ml hydrocortisone, and 5 ng/ml epidermal growth factor, supplemented with 5% horse serum. As previously described, the BC cells used have different levels of ErbB2 protein expression evaluated using the Herceptest kit scoring guidelines (score of 0: negative; score 1 + : weakly positive; score of 2: positive; score of 3 + : strongly positive) and different HER2 genomic amplification. In particular, BT474 and SKBR3 cells show high levels of ErbB2 protein (score 3 +) and HER2 genomic amplification (up to 11 copies), T47D cells show positive ErbB2 protein expression (score 2 +) with low levels of HER2 amplification (up to four copies), and ZR75.1 cells show positive ErbB2 protein expression (score 2 +) without genomic amplification [28–30].

The U937 leukemic cell line was used as a positive control for G-CSF receptor (G-CSFR) expression. It was cultured in Iscove Modified Dulbecco's Medium (Invitrogen) with 10% FBS.

To assess the effect of G-CSF on ErbB2 expression, BC cells (10^6/dish) were treated with 50 ng/ml G-CSF for up to 6 days and then subjected to Herceptest, western blot analysis, and flow cytometric analysis to quantify the ErbB2 receptor. For trastuzumab treatment, the cells were incubated with and without 10 μg/ml of trastuzumab (Herceptin; Roche Spa, Monza, Italy) and/or 50 ng/ml G-CSF for up to 6 days and analyzed for growth and apoptosis. The drug was freshly prepared every time in the culture medium.

Nine tumor samples from BC specimens that received G-CSF during adjuvant therapy were analyzed for ErbB2 expression by Herceptest before and after administration of growth factor. Table 1 summarises the schema of preoperative adjuvant treatment of selected BC patients.

Immunohistochemistry

Herceptest (Dako) was performed according to the manufacturer's instructions to detect ErbB2 expression. Formalin-fixed cells were spotted onto slides. Epitope retrieval was performed with Dako Epitope Retrival Solution (0.01 mol/l citrate buffer, pH 6.0) at 95–99°C for 4 min, followed by a period of 20 min at room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. The cells were incubated with primary rabbit polyclonal antibody anti-ErbB2 and with the polymer visualization reagent for 30 min each. After development with substrate chromogen solution for 10 min, sections were counterstained with Mayer's hematoxylin.

G-CSFR detection was performed on formalin-fixed and paraffin-embedded tumor samples obtained by surgical biopsy. Briefly, deparaffinized and rehydrated sections were incubated with rabbit polyclonal primary antibody anti-G-CSFR (Santa Cruz Biotechnology, Santa Cruz, California, USA) for 60 min and then the antibody was revealed by the DAKO ChemMate detection kit.

Western blot analysis

Cells were subjected to lysis with lysis buffer (50 mmol/l Tris–HCl pH 7.5, 250 mmol/l NaCl, 2 mmol/l ethylenediaminetetraacetic acid, 50 mmol/l sodium fluoride, 0.1 mmol/l sodium orthovanadate, 0.5% nonidet-P40, 1 mmol/l dithiothreitol, and a cocktail of protease inhibitors) for 15 min at 4°C and centrifuged at 20 000*g* for 15 min. Proteins (10 μg) were electrophoresed on 7.5% SDS-PAGE and transferred to 0.45-μm polyvinylidine diflouride membranes (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Nonspecific sites were blocked with 5% nonfat dry milk (BioRad Laboratories, Munchen, Germany). The membrane was immunoblotted with 1 μg/ml rabbit polyclonal anti-G-CSFR or anti-ErbB2 (Santa Cruz Biotechnology) and then with 1 μg/ml horseradish peroxidase-conjugated secondary

Table 1 Preoperative adjuvant chemotherapy in breast cancer patients

Patient	Regimen
1	TA × 4
2	TA × 4
3	TA × 4
4	TA × 4
5	AC × 4
6	TA × 4
7	TA × 4
8	TA × 4
9	FEC × 4

AC, adriamycine and cyclophosphamide; FEC, 5-fluorouracil, epirubicin, and cyclophosphamide; TA, taxol and adriamycine.

antirabbit antibody (Amersham) with 1% nonfat dry milk. Filter was revealed with a chemiluminescence reagent (Amersham) and exposed to an autoradiography film. For immunoprecipitation, cell lysates were incubated with anti-ErbB2 antibody and protein A for 2 h on an orbital rotor at 4°C. The immunocomplexes were spun at 4000 rpm for 3 min and washed four times with ice-cold lysis buffer. Cell lysates or immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted with antiphosphotyrosine antibody (Santa Cruz Biotechnology).

Quantitative analysis in flow cytometry

We quantified the ErbB2 receptor by flow cytometry using an immunolabeling standard, represented by beads coated with a determined amount of antigenic sites (Qifikit; Dako). The amount of antigen was expressed as the antibody-binding capacity and this gives information about the antigen density. Briefly, 2×10^5 cells were labeled with primary mouse monoclonal antibody anti-ErbB2 (Santa Cruz Biotechnology). In a separate tube, cells were labeled with irrelevant mouse monoclonal antibody. Then, cells and set-up beads and calibration beads of the kit were labeled, in parallel, with fluorescein-conjugated antimouse secondary antibody. Samples were analyzed by flow cytometer FACS Vantage (Becton Dickinson, San Jose, California, USA) and the antibody-binding capacity was calculated based on the equation of the calibration curve following the manufacturer's instructions.

Growth in soft agar

Approximately 10^4 cells/well were suspended in DMEM containing 5% (FBS) and 0.5% SeaPlaque agarose (BMA, Rockland, Maine, USA) and then overlaid with 1% agarose. The cells were treated with trastuzumab alone and/or with G-CSF. After 14 days, the cells were stained with nitro blue tetrazolium (Sigma) and colonies were counted. All experiments were performed in triplicate.

In-situ tunel assay

For apoptosis detection, we used Tunel technology based on labeling of DNA strand breaks (In-situ Cell Death Detection kit, POD; Roche Diagnostics, Mannheim, Germany). Briefly, formalin-fixed cells were rehydrated and treated with 0.2 μg/ml proteinase K (Sigma) for 5–8 min at room temperature and rinsed with Tris ethylenediaminetetraacetic acid buffer. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 10 min and the cells were washed with terminal desoxynucleotidyl transferase buffer for 5 min. Cells were permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice (4°C). They were rinsed with PBS and incubated with the labeling-reaction mixture containing fluorescein-labeled nucleotides and terminal desoxynucleotidyl transferase in a humidified chamber for 1 h at 37°C. Slides were rinsed with PBS and treated with antifluorescein antibody conjugated with peroxidase for 30 min at 37°C in a humidified chamber. After washing, diaminobenzidine (Sigma) was used for visualization. Counterstaining was performed with hematoxylin.

Statistical analysis

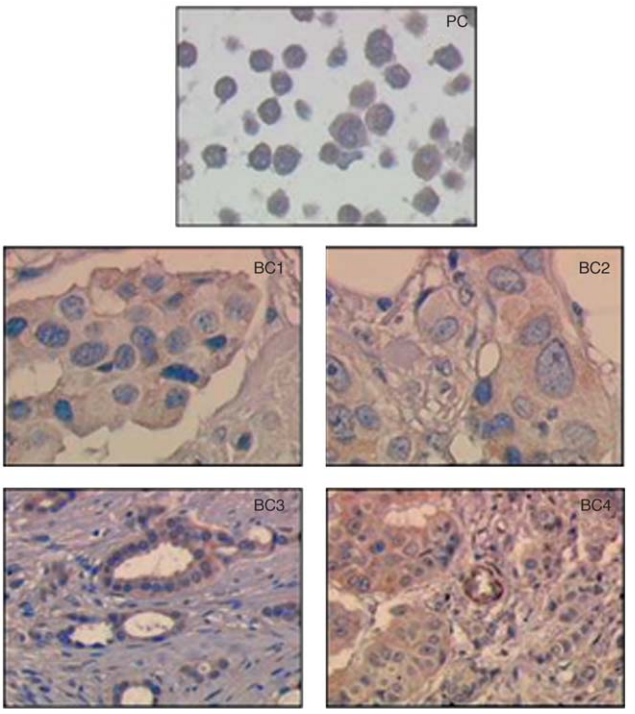
For receptor quantification, cell growth in soft agar, and apoptosis, statistical comparison of mean values was performed using the Student's *t*-test (paired and unpaired). All P values are two tailed with a significance of $P < 0.05$.

Results

Granulocyte-colony stimulating factor receptor expression on breast cancer cell lines and primary tumors

G-CSFR protein expression was determined by western blot analysis on BC cell lines, BT474, SKBR3, ZR75.1, T47D, MCF7, and MCF10A. All these cell lines express G-CSFR at different levels (data not shown). The cell surface distribution of G-CSFR was evaluated by immunohistochemistry on 10 formalin-fixed, paraffin-embedded breast tumor samples obtained after surgical operation. The G-CSFR was detected on all samples at

Fig. 1



Representative granulocyte-colony stimulating factor receptor (G-CSFR) immunostainings of breast tumors. Formalin-fixed and paraffin-embedded tumor samples were stained with anti G-CSFR and counterstained with Mayer's hematoxylin. U937 cells were used as positive control (PC) for G-CSFR expression. BC1–BC4: samples of primary breast cancer.

different levels of expression. Representative G-CSFR immunostainings are shown in Fig. 1.

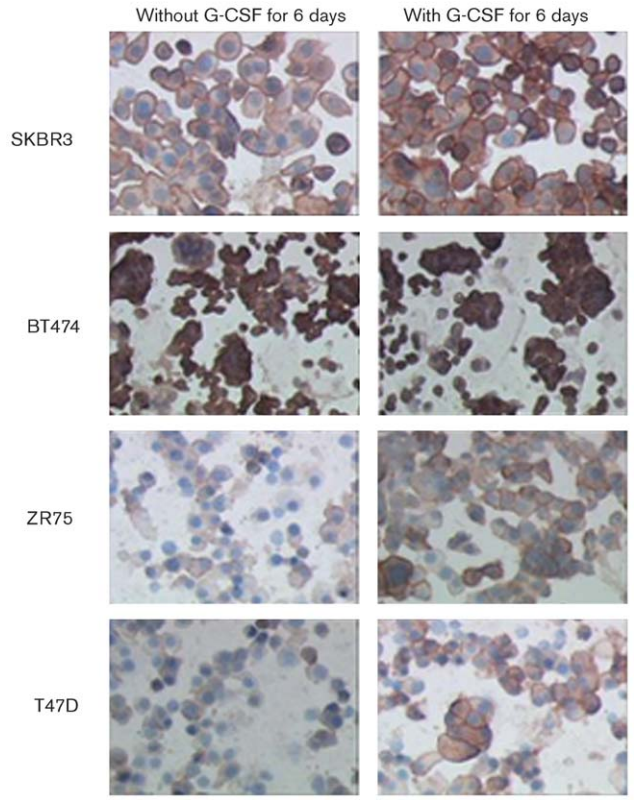
Granulocyte-colony stimulating factor increases in-vitro and in-vivo ErbB2 expression without affecting proliferation of breast cancer cell lines

We examined the effect of G-CSF on ErbB2 protein expression in BC cells. Cell exposure to G-CSF induced an increase of ErbB2 receptor in SKBR3, ZR75.1, and T47D cells as demonstrated by Herceptest (Fig. 2). This increment was not detectable on BT474 cells that already expressed a high level of ErbB2 protein. The upregulation of ErbB2 was evident after 1 day of G-CSF exposure with a pick after 3 days (data not shown). The MCF7 cell line with Herceptest score 1+ and the MCF-10A, an ErbB2 negative cell line, did not change their HER2 status upon G-CSF stimulation (data not shown). The increase of ErbB2 level was confirmed on SKBR3, ZR75.1, and T47D cells by western blot analysis (Fig. 3a). By contrast, tyrosine phosphorylation of ErbB2, observed by immunoprecipitation and immunoblotting, was not modified upon G-CSF treatment (data not shown). To better quantify the degree of ErbB2 modulation, we performed a quantitative cytometric analysis on G-CSF-treated cells. As demonstrated in Fig. 3b, the number of ErbB2 receptors per cell increased to 9, 25, and 15% on SKBR3, ZR75, and T47D cells, respectively, after G-CSF treatment. These differences were statistically significant ($P < 0.05$).

Several studies have shown that ErbB2 overexpression enhances tumor cell growth and correlates with a poor prognosis [3,14,31–33]. To assess whether the G-CSF-dependent ErbB2 modulation correlates with an increase in tumor cell growth, we performed a soft agar assay on G-CSF-treated cells. No significant increase of cell growth was found upon G-CSF treatment in cell lines in which G-CSF treatment upregulates ErbB2 expression (Fig. 4).

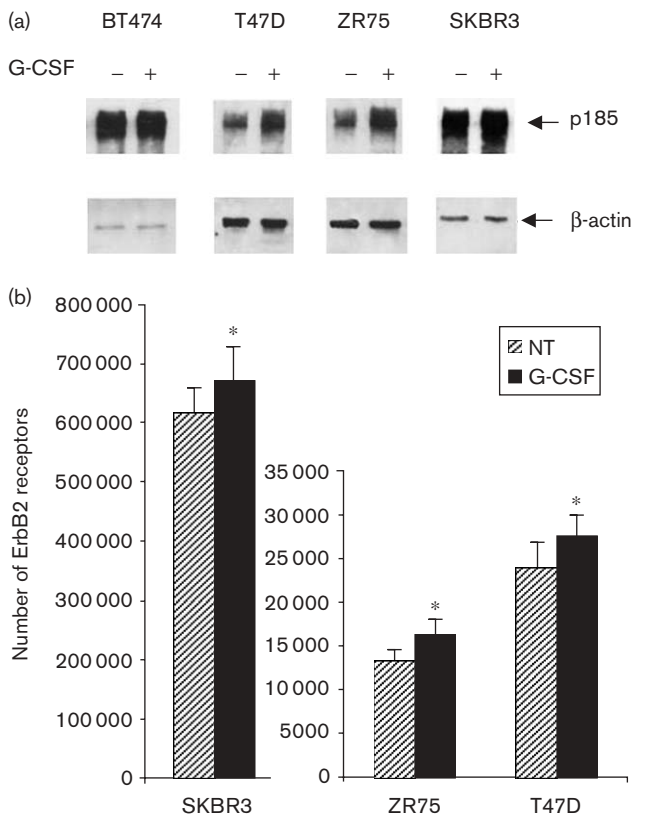
G-CSF is commonly administrated to patients after chemotherapy to increase their neutrophil counts and

Fig. 2



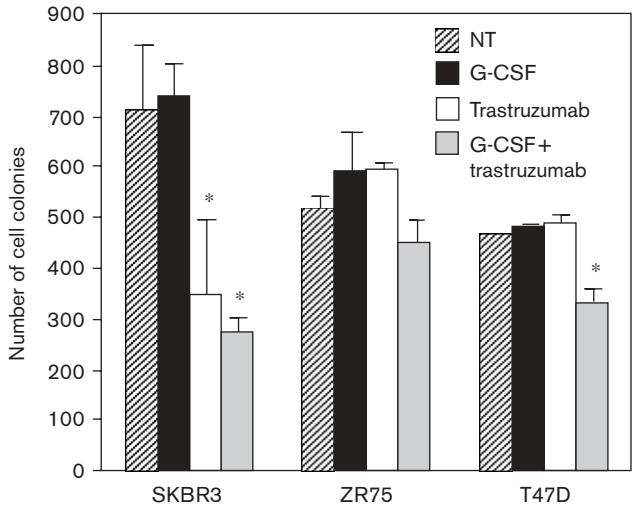
Representative Herceptest on breast cancer cell lines treated with granulocyte-colony stimulating factor (G-CSF). Breast cancer cell lines were cultured with or without 50 ng/ml G-CSF for 6 days. Cells were then fixed and immunostained with anti-ErbB2 antibody following Herceptest kit procedure.

Fig. 3



Western blot analysis on granulocyte-colony stimulating factor (G-CSF) treated breast cancer cells (a). Cells were cultured with (+) or without (–) 50 ng/ml G-CSF for 6 days. After protein extraction, ErbB2 (p185) and β-actin (as control for total protein content) immunoblotting was done. ErbB2 quantitative cytometric analysis (b). Breast cancer cells were cultured in the absence (NT) and presence (G-CSF) of 50 ng/ml G-CSF for 6 days and the number of receptors that corresponds to the antibody-binding capacity was calculated as described in Materials and Methods section. *$P < 0.05$ compared with not treated (NT) cells.

Fig. 4



Effects of granulocyte-colony stimulating factor (G-CSF) alone and in combination with trastuzumab on the growth of breast cancer cell lines: Breast cancer cells, resuspended in 0.5% SeaPlaque agarose containing Dulbecco Modified Eagle Medium (DMEM) plus 10% FCS, were overlaid on 1% agarose and incubated at the indicated culture conditions: cells cultured in the presence of DMEM plus 10% FCS (NT) added with 50 ng/ml G-CSF (G-CSF), or with 10 μg/ml trastuzumab (trastuzumab) or with the combination of either (G-CSF + trastuzumab). Colony number grown in soft agar was counted after 14 days. *$P<0.05$ compared with not treated (NT) cells.

Table 2 Herceptest scoring in breast cancer patients before and after G-CSF treatment

Patient	G-CSF before surgical treatment (months)	Herceptest before G-CSF treatment (scoring and percentage of positive cells)	Herceptest after G-CSF treatment (scoring and percentage of positive cells)
1	2	0	0
2	1	3+	3+
3	3	0	2+ (10%)
4	5	2+ (20%)	3+ (60%)
5	3	3+	3+
6	2	3+	3+
7	3	0	1+
8	2	0	1+
9	2	2+ (20%)	2+ (60%)

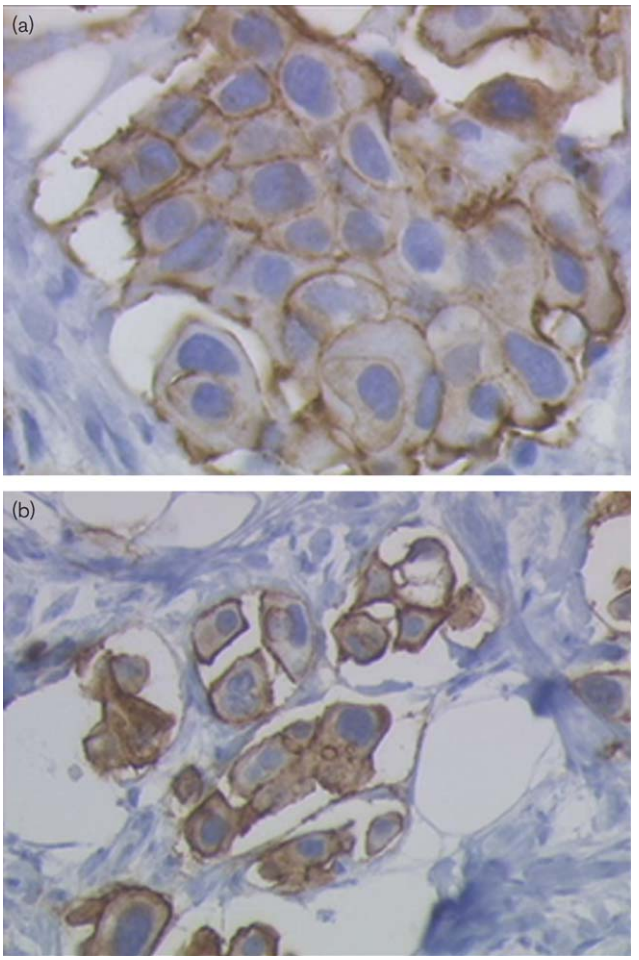
G-CSF, granulocyte-colony stimulating factor.

protect them from infection. We selected nine BC patients who received systemic administration of granulokine in their management for neutropenia treatment and we analyzed the ErbB2 expression before and after administration. An increase of ErbB2 expression, determined by Herceptest, was observed in 5/9 (55.5%) patients in terms of increase of score and percentage of ErbB2-positive cells (Table 2 and Fig. 5).

Effect of trastuzumab and granulocyte-colony stimulating factor on breast cancer cell growth

The results described above show that the amount of ErbB2 protein available on the surface of the BC cell lines

Fig. 5



Representative in-vivo increase of ErbB2 expression on breast cancer patient. Herceptest was performed in formalin-fixed tumor samples before (a, score 2+) and after (b, score 3+) granulocyte-colony stimulating factor administration.

can be increased by G-CSF treatment. Several studies demonstrated that trastuzumab causes growth inhibition on ErbB2 overexpressing BC cells [19].

To determine whether the increase of ErbB2 owing to G-CSF enhances the antiproliferative effect of trastuzumab or induces activity of trastuzumab on ErbB2 non-overexpressing cell lines, BC cells were subjected to an anchorage-independent growth assay. As shown in Fig. 4, trastuzumab was effective on ErbB2 overexpressing SKBR3 cell line (score 3+ with gene amplification) as a single agent and its antitumor activity was weakly enhanced in combination with G-CSF.

Trastuzumab alone was not effective on ErbB2 overexpressing T47D cell line (score 2+ with low level gene amplification) and on ZR75.1 cells (ErbB2 score 2+ without gene amplification). Interestingly, the

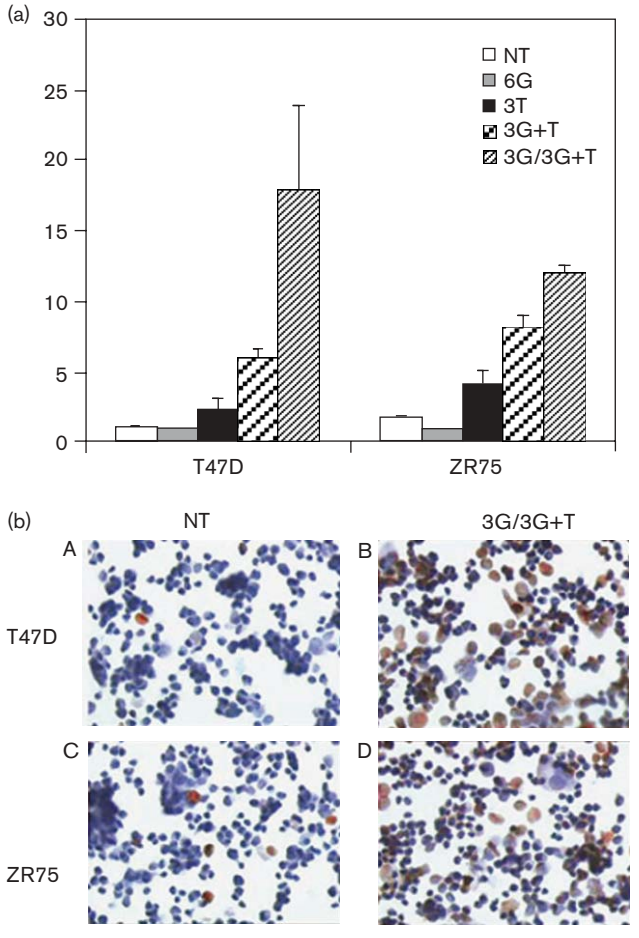
combination of G-CSF plus trastuzumab inhibits ZR75.1 and T47D colony formation in soft agar ($P < 0.05$ for T47D cells).

Effect of trastuzumab and granulocyte-colony stimulating factor on breast cancer cell apoptosis

We analyzed whether the G-CSF treatment modulates apoptosis on BC cells treated with trastuzumab for 3 days. G-CSF alone did not induce apoptosis on BC cells after a 6-day treatment (Fig. 6a). On SKBR3 cells, trastuzumab alone did not induce apoptosis (data not shown), confirming the results of other authors that have shown that growth inhibition induced by trastuzumab on this cell line *in vitro* is not owing to the induction of apoptosis, but the cell cycle arrest at G1 stage [19,20]. This effect on SKBR3 cells was not modified by the G-CSF addiction or by the G-CSF pretreatment.

On T47D and ZR75.1 cells, trastuzumab plus G-CSF induced apoptosis, and this effect was more prominent after G-CSF pretreatment (Fig. 6). The percentage of apoptotic cells was significantly increased (17.6% ± 6.2) on T47D treated simultaneously with G-CSF in combination with trastuzumab (3G + T), compared with trastuzumab alone (3T) (2.3% ± 0.4) ($P = 0.04$). Cells have been also pretreated with the growth factor for 3 days before receiving the combination of G-CSF and trastuzumab for another 3 days (3G/3G + T) enhancing the proapoptotic effect (Fig. 6a and b). This enhancement is more evident on T47D cells compared with ZR75.1.

Fig. 6



Trastuzumab in the presence of granulocyte-colony stimulating factor (G-CSF) induces apoptosis on breast cancer cells (panel a). T47D and ZR75.1 cells were treated with G-CSF alone for 6 days (6G), with trastuzumab alone for 3 days (3T), or with the combination of G-CSF and trastuzumab (3G + T), and with G-CSF for 3 days followed by the association of G-CSF and trastuzumab for another 3 days (3G/3G + T). NT, not treated cells maintained in culture in the presence of medium and 10% FBS for 6 days. Representative experiment of apoptosis induction on breast cancer cells (panel b). T47D (a) and ZR75.1 (c) cells maintained in the presence of 10% FBS; T47D (b) and ZR75.1 (d) cells treated with G-CSF for 3 days and then with the combination of G-CSF and trastuzumab for another 3 days. Cells were fixed with formalin and analyzed for apoptosis using in-situ TUNEL protocol as described in Materials and Methods section.

Discussion

Trastuzumab, an anti-ErbB2 monoclonal antibody, has in-vitro and in-vivo antitumor activity against ErbB2 overexpressing BC cells. Trastuzumab exerts its effect by growth reduction in BC cells with ErbB2 overexpression sustained by genomic amplification. In cells with low level of ErbB2 amplification, growth reduction is evident at very high doses of drug. By contrast, cells without Erb2 amplification are resistant to trastuzumab [29]. Identification of molecules able to modulate the ErbB2 expression on breast cancers with low level of the receptor might increase the efficacy of trastuzumab treatment.

In this study we demonstrated that G-CSF, a hemopoietic growth factor largely used in BC patient management, is able to increase the number of ErbB2 receptors per cell on BC cell lines with different levels of ErbB2 amplification and surface expression. The increase was more pronounced in cells with low or no ErbB2 genomic amplification. Namely, in ZR75.1, a cell line that does not present Erb2 genomic amplification, the increase was 25%; in T47D that presents low levels of HER2 amplification (up to four copies) it was 15%; and in SKBR3 cell line with high levels of HER2 genomic amplification (up to 11 copies) it was 9%. The increase of ErbB2 expression was also revealed in 55.5% of BC patients treated with G-CSF in which a modification of Herceptest score was observed comparing the tumor sample before and after growth factor administration.

It has been demonstrated that the molecular basis of ErbB2 overexpression on BC cells at the transcriptional level involves AP-2 and Ets family transcription factors [34–36]. The mechanism involved in ErbB2 modulation by G-CSF is not clear. G-CSF activates the JAK-STAT and

the Ras pathways. The activation of Ras initiates an intracellular kinase cascade, consisting of Raf, MEK, and ERK kinase [37–40], which leads to the phosphorylation of transcription factors, such as Ets proteins. Among them, the Ets transcription factor Er81 regulates the ErbB2 promoter activity [41]. We could speculate that the activation of the Ras pathway by G-CSF could induce the activation of proteins involved in ErbB2 transcription. Ongoing studies will help us to understand the relation between these two receptors.

ErbB2 overexpression has an important role in the network of cell signals controlling cell growth [42]. Potentially, enhancing ErbB2 expression may create a proliferative advantage and increase tumor aggressiveness. However, we demonstrated that G-CSF did not induce a significant increase in anchorage-independent cell growth. In-vitro studies demonstrated that in the SKBR3 cell line overexpressing ErbB2 with high level of genomic amplification, 20% of cell growth inhibition is obtained with 1 μg/ml, whereas in the ErbB2 overexpressing cells with low genomic amplification the inhibition is obtained only at high doses of trastuzumab (up to 100 μg/ml); on HER2 nonamplified cell lines 20% growth inhibition is reached only with 500 μg/ml [29]. We raised the hypothesis that the increase of ErbB2 levels produced by G-CSF administration might induce sensitivity to trastuzumab in resistant BC cells with low level of ErbB2 genomic amplification or enhance the antitumor activity of trastuzumab in ErbB2 overexpressing cells. Hence, we analyzed the effect of combination of G-CSF and trastuzumab on tumor growth and apoptosis in BC cell lines with different HER2 status. We provide evidence that a simultaneous treatment with trastuzumab and G-CSF permitted less colony formation in soft agar compared with no treatment or treatment with trastuzumab alone. The growth inhibitory effect was statistically significant on the T47D cell line, and a trend toward a decrease of colony formation was also evident on ZR75.1 cells that are considered trastuzumab resistant. Moreover, the administration of G-CSF in combination with trastuzumab induced apoptosis on T47D and ZR75.1 cell lines. On T47D cells, G-CSF pretreatment for 3 days followed by a combination of the two drugs produced up to 17% of apoptotic cells. We could explain these results by assuming that the growth factor treatment allows resistant cells to become trastuzumab sensitive probably by increasing the level of ErbB2 receptor. Cell surface ErbB2 expression upon G-CSF administration might exceed a cutoff level, over which trastuzumab is able to exert its antitumor effect.

In conclusion, taken together, our results indicate that G-CSF treatment might allow the extension of the trastuzumab antitumor activity to generally unresponsive BC cells and the potential consequences of G-CSF in-vivo administration should be further investigated.

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