Abstract. Breast cancer (BCa) is the most common cancer affecting women worldwide. Overexpression of human epidermal growth factor receptor 2 (HER2) occurs in ~20-25% of invasive ductal breast carcinomas and is associated with the more aggressive phenotype. Herceptin, a humanized antibody against HER2, is a standard therapy in HER2-overexpressing cases. Approximately one-third of patients relapse despite treatment. Therefore numerous studies have investigated the molecular mechanisms associated with Herceptin resistance. An interaction between HER2 signalling and steroid hormone receptor signalling pathways has been previously investigated, but the effect of this relationship on Herceptin resistance has never been studied. The present study analysed an impact of the steroid hormone, progesterone (PG), on Herceptin-dependent cell growth inhibition. Results indicated that Herceptin-inhibited proliferation of breast cancer cell lines overexpressing HER2 (BT474 and MCF/HER2) in 3D culture is abolished by PG. Furthermore, results demonstrated that PG led to the activation of HER2/HER3-mediated signalling. Moreover, PG treatment induced a shift of Herceptin-dependent cell cycle arrest in G1, phase towards S and G2 phases with concomitant upregulation of cyclin-dependent kinase 2 (CDK2) and downregulation of CDK inhibitor p27Kip1. These results demonstrate for the first time PG involvement in the failure of Herceptin treatment in vitro. The present observations suggest that cross-talk between PG- and HRG/HER2-initiated signalling pathways may lead to the acquisition of resistance to Herceptin in patients with BCa.

Introduction

Overexpression of human epidermal growth factor receptor 2 (HER2) occurs in approximately 20-25% of invasive ductal breast carcinomas (BCa). It is associated with increased metastatic potential and poor prognosis (1). HER2 belongs to the receptor tyrosine kinases (RTK) HER family that comprises three other members (HER1/EGFR, HER3, HER4), which require specific ligand binding for activation. In contrast, no ligand has been identified for HER2 yet. Overexpressed HER2 was found to be constitutively phosphorylated in both BCa cell lines and tumours (2). HER2 forms homodimers or heterodimers with other ligand-activated members of the HER family (3). HER2/HER3 heterodimer has been demonstrated as the most potent oncogenic unit in HER2-positive BCa (4).

Herceptin (Trastuzumab) is a humanized antibody directed against the extracellular domain of HER2 and routinely used for the treatment of HER2-overexpressing BCa patients. The mechanism of Herceptin-mediated cell death is complex and involves antibody-dependent cell-mediated cytotoxicity, induction of apoptosis, inactivation of HER2 homodimerization and abrogation of HER2-triggered cell signalling (5-7). Clinical data showed that some patients either originally do not respond to Herceptin or become resistant during the treatment (8,9). There is a growing evidence demonstrating the interaction between HER2 signalling and estrogen receptor (ER) pathway (10). PR (progesterone receptor), one of the ER-dependent genes, together with its cognate ligand-progesterone (PG), play a critical role in breast cancer development and progression (11-13). A cross-talk between steroid hormones and RTK (e.g., HER receptor)-initiated signalling has a bidirectional nature. Steroid hormone receptors may activate either RTKs or their downstream signalling pathways (14,15). Balka et al (16) demonstrated in MPA (medroxyprogesterone acetate-synthetic progestin)-induced mice mammary adenocarcinomas, an interaction between progestins- and heregulin (HRG) (HER1/EGFR and HER3 ligand)-dependent signalling. Conversely, RTK-triggered pathways are able to modulate steroid receptor’s activity (17). HER2 overexpression has been linked with resistance to endocrine therapies both in vitro and in vivo (10). Consistently, there are studies showing that ER activity can function as an escape pathway for ER+/HER2+ cells exposed to anti-HER2 treatment (18). The role of PG/PR in the process of resistance to anti-HER2 therapies remains elusive. Taking into
consideration reciprocal interactions between steroid hormone receptors and HER2-mediated signalling, we hypothesised that PG may affect anti-proliferative effect of Herceptin.

Herein we showed for the first time that PG may attenuate the efficacy of HER2-targeted anticancer compounds. We demonstrated that PG impaired Herceptin-mediated anti-proliferative action in HER2-overexpressing cell lines and led to activation of HER2/HER3-triggered signalling. Moreover, PG reversed Herceptin-induced cell cycle arrest in G0/G1 cell cycle phase with concomitant upregulation of cyclin-dependent kinase 2 (CDK2) and downregulation of CDK inhibitor p27Kip1. These findings indicate complexity of the mechanism responsible for resistance to Herceptin and suggest that targeting of multiple signalling pathways may result in better therapeutic effects.

Materials and methods

Cell lines, antibodies, reagents. BT474 (cat. no. HTB-20™) and MCF7 (cat. no. HTB-22™) cell lines were obtained from ATCC, cells from passages 86 through 106 and 71 through 91, respectively, were used in these investigations. BT474 cells were maintained in RPMI-1640 supplemented with 5 µg/ml insulin, whereas MCF7 cells were grown in DMEM. Media contained 10% of FBS and penicillin/streptomycin (100 U/ml/100 µg/ml). All cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or HyClone (Logan, UT, USA). Cells were cultured for a maximum of 3-4 months post resuscitation and regularly tested for mycoplasma contamination.

Mouse monoclonal antibody against β-actin (A5316, dilution 1:1,000) was obtained from Sigma-Aldrich. All the remaining antibodies were from Cell Signaling Technology, Inc. (Danvers or Beverly, MA, USA): Rabbit monoclonal anti-CDK2 (no. 2546, dilution 1:1,000), rabbit monoclonal anti-HER2/ErbB2 (no. 4290, dilution 1:1,000), rabbit polyclonal anti-HER2/ErbB2-Tyr877 (no. 2221, dilution 1:1,000), rabbit polyclonal anti-HER2/ErbB2-Tyr1221/1222 (no. 2249, dilution 1:1,000), rabbit monoclonal anti-HER2/ErbB2-Tyr877 (no. 2241, dilution 1:1,000), rabbit polyclonal anti-HER2/ErbB2-Tyr1248 (no. 2247, dilution 1:1,000), rabbit monoclonal anti-HER3/ErbB3 (no. 4754, dilution 1:1,000), rabbit monoclonal anti-HER3/ErbB3-Tyr1289 (no. 4791, dilution 1:1,000), rabbit polyclonal anti-HER2/ErbB2 (no. 4290, dilution 1:1,000), rabbit monoclonal anti-HER2/ErbB2 (no. 4290, dilution 1:1,000), rabbit monoclonal anti-p27Kip1 (no. 3686, dilution 1:1,000), rabbit monoclonal anti-PG (no. 3686, dilution 1:1,000). PG was purchased from Sigma-Aldrich. Herceptin was obtained from Genetech.

Western blot analysis. Cells were grown in monolayer to 60-70% confluence, scraped in cold PBS and lysed with Laemmli buffer (2X concentrated) supplemented with: 2 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM EGTA, 1 mM EDTA, 2 mM Na3P04, 5 mM NaF and 5 mM NaVO4. Samples containing equal amounts of protein per lane were loaded, resolved in SDS-PAGE and then transferred onto nitrocellulose membrane. The membranes were incubated for 1 h in 5% skimmed milk and probed overnight with specific primary antibodies at 4°C. Secondary goat anti-rabbit (A9169, 1:20,000) or rabbit anti-mouse (A9044, 1:10,000) antibodies conjugated with HRP (Sigma-Aldrich) and Western Lightning Plus-ECL (PerkinElmer, Inc., Waltham, MA, USA) were used to visualize specific proteins.

<table>
<thead>
<tr>
<th>BT474</th>
<th>% of cells in G0-G1</th>
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<tr>
<td>CTR</td>
<td>72.85±2.31</td>
</tr>
<tr>
<td>PG</td>
<td>66.47±3.80</td>
</tr>
<tr>
<td>HERCEPTIN</td>
<td>79.91±1.63</td>
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<tr>
<td>PG+HERCEPTIN</td>
<td>73.12±2.51</td>
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Cell growth in three-dimensional Matrigel. The three-dimensional cell growth assay was performed in a Matrigel matrix (BD Biosciences, Heidelberg, Germany) as previously described (19). Briefly, 1x10^5 cells were resuspended in 40 µl of Matrigel (~2 mg of total protein/ml), placed into 12-well tissue culture plates followed by 30 min incubation at 37°C for Matrigel to solidify. 3D cultures were then covered with regular medium supplemented, when appropriate, with PG (100 nM) and/or Herceptin (10 µg/ml). Media were refreshed every 3 days. To evaluate cell growth, a mean colony diameter was measured for, at least, 50 randomly chosen colonies after 10 days of culture with ZEISS PrimoVert microscope and ImageJ software and the mean colony volume was determined. Each experiment was repeated, at least, three times.

Development of HER2 overexpression in MCF7 cells. MCF7 cells were plated in 60 mm plates and grown in the monolayer to approximately 50% confluence. Medium was refreshed 1 h before transfection. Cells were transfected with 1 µg of pBABEPuro-ERBB2 plasmid (no. 40978; Addgene, Inc., Cambridge, MA, USA) (20) containing full-length HER2 cDNA coding region) in serum free DMEM applying TurboFect reagent, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Selection of MCF7 stably expressing ERBB2 was carried out in 2 µg/µl puromycin (Gibco, Grand Island, NY, USA).

Flow cytometry. Cells were grown in 12-well plates in the monolayer up to 50% of confluence and serum starved overnight. Then cells were treated with PG (100 nM) and/or Herceptin (10 µg/ml) 24 h after stimulation, cells were trypsinized, washed twice with ice-cold PBS, fixed in 70% ethanol at -20°C for 15 min, resuspended in RNaseA 1 mg/ml (EURX Ltd. Gdansk, Poland) and stained with propidium iodide (2.5 µg/ml). Cell cycle was analysed with BD LSR II flow cytometer (BD Biosciences).

Stimulation with growth factors, treatment with Herceptin, signalling analyses. For analysis of growth factors-triggered signalling, cells were serum-starved overnight before growth factors were added. Cells were stimulated with PG (100 nM), Herceptin (10 µg/ml) for indicated periods of time.

Statistical analysis. Data are expressed as means ± SD from at least three independent experiments. Comparative data were analysed with the unpaired Student's t-test using the STATISTICA software (v.10; StatSoft, Inc., Tulsa, OK, USA).
Two-sided P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PG impairs Herceptin effect on HER2-overexpressing cells growth.** To investigate the potential impact of PG on HER2-overexpressing cells response to Herceptin treatment we evaluated BT474 BCa cells (PR⁺, HER2++) growth in three-dimensional Matrigel. We found a modest (~28%) PG-triggered stimulation of cell proliferation (reflected in colony size) (Fig. 1A, left panel). As expected Herceptin significantly inhibited colonies growth (~88%, P<0.05). Importantly, Herceptin-mediated inhibition of growth was impaired by PG (Fig. 1A, right panel). To confirm these results we developed a HER2-overexpressing variant (MCF/HER2) of MCF7 BCa cells (representing luminal A subtype, ER⁺/PR⁺/HER2) by transient transfection with plasmid coding erbB2 gene (Fig. 1B). Growth analysis in three-dimensional Matrigel revealed that overexpression of HER2 resulted in sensitization of MCF7 cells to Herceptin (Fig. 1C). Although PG had modest growth stimulatory effect on both MCF7 and MCF7/HER2 cells, it clearly exerted a significant protective effect against Herceptin treatment (**P<0.05**).

**PG induces both activation and expression of HER2/HER3 signalling.** To analyse mechanisms of PG action on HER2 and HER3 function we treated BT474 cells with PG (up to 60 min) and analysed HER2/HER3 activation. It was observed that PG triggered rapid phosphorylation of Tyr1221/1222, Tyr1248 and Tyr877 of HER2 (Fig. 2A). In addition, PG induced Tyr1289 HER3 phosphorylation. Prolonged exposure of BT474 cells to PG (up to 72 h) showed that PG not only regulated the activation of HER2 and HER3 but also caused a gradual increase in their expression (Fig. 2B). In addition, PG enhanced the expression of heregulin β-1, a HER3 ligand, the binding of which is known to promote HER2/HER3 heterodimerization (21). Heregulin β-1 reached the peak of expression after 48 h of exposure to PG (Fig. 2B).
PG reverses Herceptin-induced cell cycle arrest. To further assess the impact of PG on cell response to Herceptin, cell cycle was analysed. Herceptin promoted the accumulation of cells in G<sub>0</sub>/G<sub>1</sub> phase (Fig. 3A; Table I), which was reverted by the PG treatment (observed as a shift towards S and G<sub>2</sub>/M phase). Analysis of PG-mediated mechanism of cell transition through G<sub>1</sub> to S demonstrated that PG attenuated both Herceptin-induced upregulation of p27<sup>Kip1</sup> and downregulation of CDK2 (Fig. 3B). These findings indicate that Herceptin-triggered cell cycle arrest at G<sub>1</sub> phase, which is most likely mediated by p27<sup>Kip1</sup>, is abrogated by PG.

Discussion

HER2 gene amplification and protein overexpression have been found to be an adverse prognostic factor in invasive ductal breast cancer. Herceptin, a monoclonal antibody directed against domain IV of HER2, was approved in 1998 for the treatment of HER2-amplified cancers (8,9). Despite the undisputed benefits of Herceptin-based therapy, clinical data show that the development of resistance to the drug remains an unsolved problem. Herceptin anticancer mechanism is complex and not fully elucidated. It has been well documented that resistance to Herceptin arises from the activation of alternative pathways, including ER-dependent signalling, which becomes the dominant driver of cell proliferation and survival (22-25). Wang et al (18) demonstrated that in ER<sup>+</sup>/HER2<sup>+</sup> tumor cells, increased expression of ER as well as its downstream target Bcl2, was associated with resistance to anti-HER2 therapy. ER was proved to enhance PR expression (26) and physically interact with PR, which promoted breast cancer cells proliferation (27). As ER and PR share similar signalling pathways (28), we hypothesized that PR activity may affect Herceptin anti-proliferative action. Herein, we demonstrated for the first time that Herceptin-mediated growth inhibition was significantly impaired by PG. We observed that a short stimulation with PG (up to 60 min) led to HER2/HER3 activation in BT474 cells. On the other hand, prolonged stimulation (up to 72 h) induced not only the expression of both receptors (i.e., HER2 and HER3) but also that of heregulin, a ligand for HER3. Our results are in accordance with the data presenting upregulation of heregulin in MPA (synthetic PG)-induced mammary tumours (16) and its impact on cell proliferation. Taken together, it can be speculated that PG action towards the anti-proliferative effect of Herceptin may involve PG-promoted increase of expression of heregulin, which, by binding to HER3, induces HER2/HER3 heterodimerization and subsequent activation of downstream signals. Our data demonstrated that PG also abolished Herceptin-mediated cell-cycle arrest in G<sub>0</sub>-G<sub>1</sub> phase. PG-promoted cell shift towards S and G<sub>2</sub>/M phase was observed with a concomitant upregulation of CDK2 and downregulation of p27<sup>Kip1</sup>. There seems to be a reciprocal regulation of PR-CDK2 activities, as CDK2 was shown to phosphorylate PR (8 out of 14 PR phosphorylation sites are known to be targeted by CDK2) (29,30) and increase PR transcriptional function (31).

Steroid hormones play a critical role in breast carcinogenesis (32-34). Although the relationship between the level of circulating estrogen/PG and breast cancer risk has been extensively studied, the effect of steroids on the efficacy of anti-HER2 BCa therapy has not been greatly explored. Our findings provide support for the hypothesis that, in steroid hormone receptors-positive BCa, acquisition of resistance to Herceptin might be triggered by PG. This finding seems to be important especially for premenopausal women, who are exposed to periodically increased level of PG. Our results
References


3. Graus-Porta D, Beeri RR, Duly JM and Hynes NE: ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 16: 1647-1655, 1997.


12. Soyle S, Kamila Kitowska has Ministry of Science and Higher Education from science funds (grant agreement no 316094-MOBI4Health project) and Polish National Science Centre -UMO-2012/06/M/NZ3/00023 gift from Matthew Meyerson (Dana-Farber Cancer Institute).


