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Experimental Cell Research 304 (2005) 604–619

Experimental Cell Research

www.elsevier.com/locate/yexcr

Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation

Simone Diermeier[a], Gábor Horváth[b], Ruth Knuechel-Clarke[c], Ferdinand Hofstaedter[a], János Szöllősi[b,d], Gero Brockhoff[a,*]

[a]*Institute of Pathology, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany*
[b]*Department of Biophysics and Cell Biology, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, Hungary*
[c]*Institute of Pathology, University of Aachen, Germany*
[d]*Cell Biophysical Research Group of Hungarian Academy of Sciences, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, Hungary*

Received 27 July 2004, revised version received 7 December 2004
Available online 21 January 2005

Abstract

Background: Growth factors and Herceptin specifically and differentially modulate cell proliferation of tumor cells. However, the mechanism of action on erbB-receptor level is incompletely understood. We evaluated Herceptin's capacity to modulate erbB-receptor activation and interaction on the cell surface level and thereby potentially impair cell proliferation of HER2/neu (c-erbB2) overexpressing breast cancer cells, both in the presence and absence of relevant growth factors. *Methods*: BT474 and SK-BR-3 breast cancer cell lines were treated with Epidermal Growth Factor (EGF), Heregulin, and with Herceptin in different combinations. Kinetics of cell proliferation were evaluated flow cytometrically based on BrdU-labeling. Fluorescence Resonance Energy Transfer, ELISAs and phosphorylation site specific Western Blotting was performed to investigate erbB-receptor interaction and activation. *Results*: EGF induced EGFR/EGFR and EGFR/c-erbB2 interactions correlate with stimulation of cell proliferation in BT474 cells. Both homo- and heterodimerization are considerably less pronounced in SK-BR-3 cells and heterointeraction is additionally reduced by EGF treatment, causing inhibition of cell proliferation. Heregulin stimulates cell proliferation extensively in both cell lines. Herceptin drives BT474 cells more efficiently into quiescence than it does with SK-BR-3 cells and thereby blocks cell cycle progress. In SK-BR-3 Herceptin treatment causes c-erbB2 phosphorylation of Y877 and Y1248, EGF induces Y877 and Y1112 phosphorylation. The Y1112 phosphorylation site, activated by EGF in SK-BR-3 cell, is bypassed in BT474. In addition the inhibitory capacity of Herceptin on BT474 and SK-BR-3 cell proliferation depends on the presence and absence of growth factors to a various extent. *Conclusion*: The growth inhibitory effect of Herceptin on c-erbB2 overexpressing breast cancer cells is considerably modulated by EGFR coexpression and consequently EGFR/c-erbB2 homo- and heterointeractions, as well as the presence or absence of growth factors. C-erbB2 overexpression alone is insufficient to predict the impact of growth factors and antibodies on cell proliferation. The optimization and specification of therapeutic approaches based on erbB-receptor targeting requires to account for EGFR coexpression as well as the potential presence of erbB-receptor relevant growth factors.

Keywords: Breast cancer treatment; erbB-receptors; HER2/neu; EGFR; Herceptin; Receptor interaction; FRET; Cell cycle; Proliferation

Introduction

The detection of HER2/neu (subsequently named c-erbB2) gene amplification via fluorescence-in-situ-hybridization (FISH) and alternatively the immunohistochemical verification of receptor protein overexpression are estab-

Abbreviations: a.u., arbitrary units; BSA, bovine serum albumine; EGF, Epidermal-Growth-Factor; EGFR, Epidermal-Growth-Factor-Receptor; Herc, Herceptin; HRG, Heregulin; Hoe, Hoechst33258; PY, phosphotyrosine; Y, tyrosine.

* Corresponding author. Fax: +49 941 944 6602.
E-mail address: Gero.Brockhoff@klinik.uni-regensburg.de (G. Brockhoff).

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doi:10.1016/j.yexcr.2004.12.008

lished diagnostic tools to evaluate breast cancer patients for Herceptin therapy [1–4]. If the c-erbB2 receptor is overexpressed, patients can benefit from this therapy in respect of prolongation of disease-free and overall survival [5,6]. Immune targeting of tumor cells, inhibition of cell proliferation, and induction of apoptosis are factors demonstrably contributing to the anti-tumor effect mediated by Herceptin [7]. However, even if c-erbB2 is overexpressed, the therapeutic efficacy of Herceptin treatment varies individually and is difficult to predict. Moreover, the signal transduction mechanism triggered by Herceptin on the erbB-receptor level is not understood in detail.

The c-erbB2 receptor, utilized as a therapeutic antibody target, is a member of a receptor family comprising c-erbB1 (EGFR), c-erbB2, c-erbB3, and c-erbB4. These highly homologue receptor tyrosine kinases (RTK) have a strong potential to assemble homo- and heterodimers upon extracellular growth factor binding and activation, resulting in extensive signal diversification [4]. Communication among erbB-receptors substantially increases the efficiency and diversity of signal transduction [8] because signal transmission across the cell membrane is mediated by lateral receptor interaction rather than by activation of just a single erbB-receptor type. Multiple erbB-receptor homo- and heterodimers trigger consecutive intracellular signaling and thereby induce a specific cellular response, for example, the stimulation or inhibition of proliferation. Due to these complex interactions, it is difficult to predict the cellular response to Herceptin and other erbB-receptor ligands. Some studies have demonstrated that, instead of targeting a single receptor, a therapeutic approach, specifically aiming a particular subset of coexpressed erbB-receptors, might augment the therapeutic effectiveness in terms of inhibited tumor growth [7,9]. The most extensively investigated strategies evaluated in preclinical settings combine the anti-erbB-receptor antibody based approach with antisense- or siRNA-technology against erbB-receptor transcripts [10], or with selective inhibition of erbB-receptor kinase activity [11]. The combination of conventional chemotherapy with Herceptin is part of clinical practice [12–15].

Although it is known that c-erbB2 belongs to a complex regulated receptor system, altogether triggering the initial events for subsequent signal transduction upon ligand binding (growth factors, anti-erbB-receptor antibodies), the coexpression profile of erbB-receptors is usually not characterized in pathological diagnosis. However, targeting multiple erbB-receptors will provide an exceptional strategy for an effective cancer therapy [16,17], but only little is known about the diagnostic and therapeutic importance of coexpressed erbB-receptors at high and low levels.

Here, we investigated the effect of Herceptin on proliferation of tumor cells, genotypically characterized by c-erbB2 gene amplification and corresponding protein overexpression [18]. We focused our study on BT474 and SK-BR-3 breast cancer cell lines, representing excellent tumor cell models with different EGFR/c-erbB2 coexpression patterns: BT474 cells show c-erbB2 overexpression but low levels of other erbB-receptors, whereas aside from c-erbB2 overexpression in SK-BR-3 the EGFR content is simultaneously high [19]. We evaluated the potential inhibitory effect of Herceptin on BT474 and SK-BR-3 cell proliferation both in the presence and absence of Epidermal Growth Factor (EGF) and Heregulin (HRG). Using the flow cytometric Foerster-type Fluorescence Resonance Energy Transfer (FRET) technique [20,21], we found substantially different ligand induced EGFR and c-erbB2 homo- and heterointeraction resulting in a cell type specific phosphorylation pattern of the c-erbB2 receptor. Herceptin inhibits cell proliferation by driving cells into quiescence more effectively when EGFR content is low. A high EGFR content renders the tumor cell line less susceptible to Herceptin indicating that the EGFR/c-erbB2 ratio plays a key role in lateral signal transduction even in the presence of c-erbB2 overexpression. The simultaneous evaluation of c-erbB2 and EGFR expression may improve erbB-receptor based oncological diagnosis, most likely affording a better patient stratification.

Materials and methods

Cell culture and siRNA transfection

The human breast cancer cell lines BT474 and SK-BR-3 were purchased from the European Collection of Animal Cell Cultures (ECACC) and routinely grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA), supplemented with 5% fetal calf serum (FCS) (Sigma-Aldrich, Taufkirchen, Germany). Cells were seeded at cell densities of 2×10^5 cells per T75 tissue flask (Greiner, Bio-One, Frickenhausen, Germany) and maintained at 37°C in humidified atmosphere of 5% CO_2. Culture medium was refreshed every 2 days replacing all reagents for cell stimulation or labeling if indicated. Cells were routinely grown for 7 days, then washed with phosphate-buffered saline (PBS, pH 7.4, Biochrom, Berlin, Germany) and detached from culture flasks by 0.05% trypsin/0.02% EDTA treatment for 3 min at 37°C in PBS. When cell proliferation was analyzed the FCS supplement was reduced to 2% 24 h before the cells were growth factor treated. For cell lysis and Fluorescence Resonance Energy Transfer (FRET) experiments, FCS was deprivated completely 24 h before growth factor addition.

Transfection with siRNA was carried out with Oligofectamine (Invitrogen, Karlsruhe, Germany), according to the manufacturer's specifications. Chemically synthesized siRNAs were kindly provided by P. Nagy and used at a concentration of 100 nM according the transfection procedure published elsewhere [10]. Sequence details are listed in Table 1.