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β-Heregulin impairs EGF induced PLC-γ1 signalling in human breast cancer cells

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ABSTRACT

The interplay of ErbB receptor homo- and heterodimers plays a crucial role in the pathology of breast cancer since activated signal transduction cascades coordinate proliferation, survival and migration of cells. EGF and β-Heregulin are well characterised ligands known to induce ErbB homo- and heterodimerisation, which have been associated with disease progression. In the present study, we investigated the impact of both factors on the migration of MDA-NEO and MDA-HER2 human breast cancer cells. MDA-NEO cells are positive for EGFR and HER3, while MDA-HER2 cells express EGFR, HER2 and HER3. Cell migration analysis revealed that β-Heregulin potently impaired EGF induced migration in both cell lines. Western blot studies showed that both ErbB receptor and PLC-γ1 tyrosine phosphorylation levels were diminished in EGF and β-Heregulin co-treated MDA-NEO and MDA-HER2 cells, which was further correlated to a significantly impaired calcium influx. Our data indicate that EGF and HRG may interfere with each other for receptor binding and dimerisation, which ultimately has an impact on signalling outcome.

1. Introduction

Members of the ErbB family of receptor tyrosine kinases, including EGFR, HER2 (c-erbB-2; HER2/*neu*), HER3 (erbB-3) and HER4 (erbB-4), are not only involved in a plethora of physiological processes, but do also play a crucial role in the pathology of many cancer types, including lung, breast, gastric, pancreatic and colorectal carcinoma (for review see: [1–3]). They belong to the group of type I receptor tyrosine kinases [4] that dimerize upon ligand binding concomitant with intracellular cross-phosphorylation of tyrosine residues within the cytoplasmic receptor domain and induction of a variety of signal transduction cascades that are involved in several cellular processes including proliferation, migration and/or survival (for review see: [1–3]). To date, about 11 ligands, like EGF, TGF-α or Heregulins (also named Neuregulins), have been identified that could bind to 26 possible ErbB homo- and heterodimers ultimately resulting in 611 potential ligand-activated dimers (excluding HER2/HER2, which has no ligand, and HER3/HER3, which lacks protein kinase activity) [3] revealing the rather complex biology of ErbB receptors.

ErbB receptors play a crucial role in the development and progression of breast cancer [5]. HER2 overexpression and/or gene amplification is found in nearly 30% of breast cancers, which are classified as HER2 rich [6]. Such breast cancers are commonly associated with a very poor prognosis of the afflicted patients due to shorter disease-free intervals, increased risk of metastasis, and resistance to many types of therapy [5,7,8]. It is well recognised that HER2 is the preferred heterodimerisation partner of all other ErbB family members [9], whereby HER2 preferentially forms heterodimers with EGFR and HER3 [10]. Co-expression of EGFR and HER2 as well as the presence of phosphorylated HER2 has been associated with enhanced formation of distant metastasis and hence a markedly reduced disease-free survival rate [11]. In this context, we have already demonstrated that the motogenic phenotype of human breast cancer cells, which is mandatory for metastasis formation, is determined by EGFR/HER2 heterodimers and activation of phospholipase C-γ1 (PLC-γ1) signalling [12,13].

In addition to EGFR and HER2, several lines of evidence revealed an

Abbreviations: DAG, diacylglycerol; FCS, foetal calf serum; IP3, inositol-1,4,5-trisphosphate; MDA-HER2, MDA-MB-468-HER2; MDA-NEO, MDA-MB-468-NEO; MDA-PM, MDA-MB-468-PM; PI3K, phosphatidyl-inositol-3-kinase; PIP2, phosphatidyl-4,5-bisphosphate; PIP3, phosphatidyl-3,4,5-trisphosphate; PLC-γ1, phospholipase C-γ1; Proximity Ligation Assay, PLA; SPC, signals per cell

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important role of HER3 in breast cancer pathogenesis and progression. HER2 and HER3 form the most active heterodimer of the ErbB family causing a strong activation of the phosphatidyl-inositol-3-kinase (PI3K)/AKT pathway, thereby efficiently transducing proliferatory and survival signals into the cells [14]. Because of that, the HER2/HER3 heterodimer has been referred to as an oncogenic unit that drives the proliferation of breast cancer cells [15]. In addition to different cellular functions, like cell survival, growth, proliferation, angiogenesis, and migration [16–18] PI3K/AKT signalling is also mandatory for PLC-γ1 activation [19,20]. Activation of PI3K results in the production of PIP3, which acts as a PLC-γ1 anchor molecule, thus recruiting PLC-γ1 to the plasma membrane in close proximity to growth factor receptor tyrosine kinases [19,20]. In this context, we have already demonstrated the interplay of HER2/HER3/PI3K and EGFR/HER2/PLC-γ1 signalling in breast cancer cell migration and dissemination [21].

β-Heregulin (also named Neuregulin-1β) is another growth factor that has been associated with breast cancer disease progression and even therapeutic resistance, mainly through induction of PI3K/AKT signalling pathway [22,23]. Smirnova et al. demonstrated that overexpression of HER3 in mammary tumour cells was further significantly correlated to an enhanced chemotaxis to β-Heregulin and an overall metastatic potential of the breast tumour cells both in vitro and in vivo [18]. Moreover, several studies revealed that β-Heregulin together with HER3 signalling could overcome the inhibitory effects of compounds targeting EGFR and HER2, like gefitinib and lapatinib, in human HER2 positive breast cancer cells [24–26].

In the present study we investigated the impact of β-Heregulin together with EGF on MDA-MB-468-NEO (MDA-NEO) and MDA-MB-468-HER2 (MDA-HER2) human breast cancer cells regarding cell migration. Interestingly, our data show that β-Heregulin induced signalling interferes with EGF induced signalling, thereby impairing the EGF induced migration of the cells.

2. Material and methods

2.1. Cell culture

MDA-NEO human breast cancer cells and MDA-HER2 human breast cancer cells were derived from the parental HER2 negative, but EGFR overexpressing breast cancer cell line MDA-MB-468, by stable transfection with either a neomycin resistance vector (MDA-NEO) or a HER2 expression vector (MDA-HER2), respectively [12,13,21]. MDA-NEO cells are positive for EGFR and HER3 expression, whereas EGFR, HER2 and HER3 are expressed by MDA-HER2 cells. MDA-NEO and MDA-HER2 cells were cultured in DMEM media (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% foetal calf serum (FCS) (Biochrom GmbH, Berlin, Germany), 10 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich, Taufkirchen, Germany), and 400 μg/ml G418 (Biochrom GmbH, Berlin, Germany) in a humidified atmosphere at 37 °C and 5% CO_2.

2.2. Pull-down assay

MDA-NEO and MDA-HER2 cells (2×10^6) were plated in 10 cm^2 cell culture dishes (Sarstedt, Nümbrecht, Germany) and were cultured for two days. Thereafter, cells were incubated overnight in starvation media (complete media lacking FCS) and were then stimulated with 100 ng/ml EGF (Sigma-Aldrich, Taufkirchen, Germany), 100 ng/ml β-Heregulin (Sigma-Aldrich, Taufkirchen, Germany) or a combination of both for different time points (0 min, 3 min, 5 min, 15 min, 30 min, and 60 min). Cells were washed twice with ice-cold PBS followed by addition of 5 ml of ice-cold biotin solution containing 200 μg/ml EZ-link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific, Bonn, Germany). Plates were put on an orbital shaker and were gently agitated for 10 min at 4 °C. Cells were washed twice with ice-cold PBS and then 5 ml of quenching solution (0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM glycine) was added and cells were incubated for additional 10 min. Cells were washed again twice with ice-cold PBS. Cells were lysed in Pierce™ IP Lysis Buffer (Thermo Fisher Scientific, Bonn, Germany) supplemented with protease and phosphatase inhibitors and incubated for 15 min on ice followed by centrifugation (13,000 rpm) to collect the supernatant. The total protein concentration of the samples was determined by using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany) following the manufacturers' instructions. Biotin-labelled proteins were affinity purified by Streptavidin magnetic beads (Thermo Fisher Scientific, Bonn, Germany). Streptavidin magnetic beads were washed once in TBS-T (TBS with 1% Tween 20 (v/v; Sigma-Aldrich, Taufkirchen, Germany) and then incubated overnight at 4 °C with samples (200 μg protein). Subsequently, beads were washed thoroughly three times with TBS-T, resuspended in 25 μl 2× Laemmli Sample Buffer and boiled for 7 min at 95 °C.

2.3. Western blot analysis

MDA-NEO and MDA-HER2 cells (2×10^5/20 μl), respectively, were treated for 1 min, 2 min, and 5 min with 100 ng/ml EGF (Sigma-Aldrich, Taufkirchen, Germany), 100 ng/ml β-Heregulin (Sigma-Aldrich, Taufkirchen, Germany), or a combination of both. Subsequently, 10 μl 3× Laemmli buffer was added and samples were lysed for 10 min at 95 °C. Cell lysates were separated by 8% or 10% SDS-PAGE and subsequently transferred to PVDF nitrocellulose membranes (Merck Millipore, Darmstadt, Germany) under semi-dry conditions. Membranes were either blocked with 10% (w/v) non-fat milk powder (Applichem, Darmstadt, Germany) or 5% BSA (Sigma-Aldrich, Taufkirchen, Germany) in TBS-T. Bands were visualised by using the Pierce ECL Western blot substrate (Thermo Fisher Scientific, Bonn, Germany) as recommended by the manufacturers' instructions and the Aequoria Macroscopic Imaging System and the HoKaWo imaging acquisition software (Hamamatsu Photonics Germany, Herrsching am Ammersee, Germany). Bands of interest were cropped from Western blot data either using ImageJ (https://imagej.nih.gov/ij/) or GIMP (https://www.gimp.org) and were grouped in a single file using Inkscape (https://inkscape.org). Primary and secondary antibodies used in this study are summarised in Table 1. ImageJ (https://imagej.nih.gov/ij/) was used for densitometric analysis of Western blots.

2.4. Flow cytometry based measurements of changes in intracellular calcium levels

Changes in the intracellular calcium levels of MDA-NEO and MDA-HER2 human breast cancer cells were determined as referred to Gergely et al. [27] and as described previously [21,28,29]. In brief, cells (5×10^5) were stained with Fluo-4 (Thermo Fisher Scientific, Bonn, Germany) prior to calcium measurements, which were performed using a FACScalibur flow cytometer (Becton Dickenson, Heidelberg, Germany). Acquisition was paused after 50 s for 15 s to add the substance of interest (10 μM Ionomycin (Sigma-Aldrich, Taufkirchen, Germany), 100 ng/ml EGF, 100 ng/ml β-Heregulin or a combination of both). The tube was mixed and acquisition was continued for a total of 204.80 s. Data analysis was performed by determining the mean fluorescence intensity (MFI) of 10 s intervals by using the WinMDI software tool (https://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). The mean MFI of the first 50 s of each calcium measurement without a stimulus served as a baseline and was set to 100%. The mean MFI of the first 30 s after stimulation was defined as the rate of calcium influx, which was calculated in relation to the baseline level.

2.5. Cell migration studies

Cell migration studies to analyze the locomotory activity of MDA-MEO and MDA-HER2 human breast cancer cells within a three-dimensional collagen matrix were performed as described previously [21,30]. In brief, 50 μl of a cell suspension containing 4×10^4 cells was