Caveolin-1 as a Potential Causative Factor in the Generation of Trastuzumab Resistance in Breast Cancer

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INTRODUCTION

Tyrosine Kinase Oncogenes and Cancer

Tyrosine kinase oncogenes are formed as a result of mutations that induce constitutive kinase activity [1]. Despite differences in structure, normal function and sub cellular location, many of the tyrosine kinase oncogenic signals through the same pathways, transmitting extracellular signals to the inside of the cell, thereby controlling cellular events such as proliferation, differentiation, and cell death [1-2]. Hence, they represent excellent potential drug targets. For instance, aberrant activation of the human epidermal growth factor (EGFR) family of receptor tyrosine kinases occurs frequently in many malignancies, including breast, lung, and brain cancer. The epidermal growth factor family consists of four receptor genes and at least eleven ligands, several of which are produced in different protein forms [2].

Epidermal Growth Factor Receptors

The mammalian EGFR family comprises four receptors (EGFR/ErbB1, ErbB2, ErbB3, and ErbB4), which are derived from a series of gene duplications early in vertebrate evolution and are 40%—45% identical [3]. ErbB receptors associations results in increased signaling potency through several means, including increased ligand affinity, increased coupling efficiency to signaling molecules, and decreased rate of receptor internalization. The importance of heterodimer-mediated signaling in normal development is obvious from studies in genetically modified mice. This is particularly true for ErbB2 -ErbB3 and ErbB2 - ErbB4 heterodimers [1-4]. Loss of ErbB2 or ErbB3 has a similar impact on neuronal development, whereas loss of ErbB2 or ErbB4 has major effect on heart development[7].

ErbB Ligands

ErbB ligands act in a paracrine or autocrine fashion. Whereas paracrine ErbB ligands are derived from stromal cells, autocrine ErbB ligands are produced as transmembrane precursors that are subsequently cleaved by proteases to be released as soluble ligands when cells are stimulated [6]. At least 10 ErbB ligands are known and are divided into three groups with respect to binding specificity. The first group includes EGF, amphiregulin (AR) and transforming growth factor (TGF), which bind specifically to ErbB1. The second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which show dual specificity, binding to both ErbB1 and ErbB4 [5-6]. The third group includes neuregulins (NRGs). They are composed of two subgroups. NRG1 and NRG2 bind specifically to both ErbB3 and ErbB4, whereas NRG3 and NRG4 bind specifically to only ErbB4.None of these ErbB ligands bind and activate ErbB2.



Fig1: ErbB family receptors and their preferred combinations.

A second member of the ErbB family, ErbB2/HER2

The *ErbB2* gene was initially identified as an oncogene named *neu* in NIH3T3 cells [2-5]. Soon after, several groups, revealed that this gene was the second member of the EGFR family, encodes a 175-kDa transmembrane glycoprotein that is highly similar to EGFR. Initial studies showed that this gene was amplified in human cancer cell lines and tissues, indicating its importance in human cancer[7]. This finding prompted the extensive studies of ErbB2 in human cancer.ErbB2, which has no ligand binding domain, phosphorylates its substrates independently of ligand binding, resulting in hyperproliferation [8]. Since ErbB2 extracellular domain is always in the open conformation, ErbB2 is the preferred binding partner of all ErbB receptors even as a monomer. Gene amplification and overexpression of its protein product are frequently observed in various types of human cancer, especially in breast cancer. [3-5]

Signaling potential of ErbB2 receptors in breast cancer

A wealth of clinical data has demonstrated that ErbB receptor tyrosine kinases, in particular ErbB2, have roles in human mammary cancer development [8]. Overexpressed ErbB2 is constitutively phosphorylated in breast cancer cells and it has been observed that targeting overexpressed active ErbB2 results in efficient inhibition of breast cancer cell proliferation, which proceeds via inhibition of intracellular signaling pathways and directly targets various members of the cell cycle machinery[9]. Interestingly, expression of ErbB3 is seen in many tumors that express ErbB2, including breast, bladder and others. Furthermore, in many ErbB2-overexpressing breast tumors, ErbB3 has elevated levels of phosphotyrosine. ErbB3

itself has impaired tyrosine kinase activity and needs a dimerization partner to become phosphorylated and acquire signaling potential [7-10]. Patients with ErbB2-overexpressing breast cancer have substantially lower overall survival rates and shorter disease-free intervals than patients whose cancer does not overexpress ErbB2. Moreover, overexpression of ErbB2 leads to increased breast cancer metastasis[11]. The important roles of ErbB2 in cancer progression render it a highly attractive target for therapeutic interventions of breast cancer. ErbB2 signals mainly through Akt and MAPK[10-12], pathways to regulate cell proliferation, migration, differentiation, apoptosis, and cell motility.

PI3K-Akt pathway

PI3K, a heterodimer composed of a p85 regulatory subunit and p110 catalytic subunit, is activated by at least two ErbB-related pathways. Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits Akt and phosphatidylinositol-dependent kinase 1 (PDK1) and activates PDK1. PDK1 phosphorylates and activates Akt. The tumor-suppressor phosphatase with tensin homology (PTEN) dephosphorylates PIP3 into PIP2 and inhibits PI3K–Akt pathway activation . Activated Akt phosphorylates many target proteins associated with cell survival, proliferation (increased cell number), and growth (increased cell size). In addition, Akt promotes angiogenesis through vascular endothelial growth factor (VEGF) and hypoxiainducible factor-1 (HIF-1)

MAPK pathway

GRB2, an adaptor protein, binds to ErbB receptors either indirectly through Shc or directly to the phosphorylated tyrosine residues. GRB2, with or without Shc later recruits Ras and activates the MAPK pathway, inducing cell proliferation, migration, differentiation and

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angiogenesis. Recent analysis using MCF10A cells showed that Shc is required for the inhibition of apoptosis and for paclitaxel resistance.



Fig 2: Ligand induced ErbB signalling in tumor cells

ErbB2 as a therapeutic target

Several exciting strides have been made to develop ErbB2-targeting strategies that block ErbB2 expression or function.

Antibody-based agents: *Trastuzumab*: One of the ErbB2 targeting therapies is the administration of a humanized monoclonal antibody Trastuzumab, which binds to juxta membrane domain of ErbB2[13]. Clinical trials have shown that Trastuzumab inhibits breast cancer cell proliferation by the induction of cell cycle arrest at the G1 phase of the cell cycle. Furthermore, the Fc γ portion of Trastuzumab is reported to elicit antibody-dependent cell mediated cytotoxicity (ADCC) [13-15] which probably is the dominant therapeutic activity of

Trastuzumab. However, in most cases, the levels of the ErbB2 surface expression on tumor cells are crucial for a potent and sustained therapeutic response to Trastuzumab.

Pertuzumab: The recombinant humanized HER2 monoclonal antibody pertuzumab (OmnitargTM, 2C4, Genentech) represents a new class of drugs called dimerization inhibitors; these have the potential to block signaling by other HER family receptors, as well as inhibiting signaling in cells that express normal levels of HER2. Pertuzumab sterically blocks dimerization of HER2 with EGFR and HER3, inhibiting signaling from HER2/HER3 and HER2/EGFR heterodimers [14-15].

Lapatinib: Lapatinib (TykerbTM, GSK572016, formerly <u>GW572016</u>; GlaxoSmithKline, Research Triangle Park, NC, USA) is a dual tyrosine kinase inhibitor targeted against both EGFR and HER2 [18]. In comparison to other tyrosine kinase inhibitors in clinical trials (for example, gefitinib, erlotinib), interaction of lapatinib with EGFR and HER2 is reversible, similar to other agents, but dissociation is much slower, allowing for prolonged downregulation of receptor tyrosine phosphorylation in tumor cells[17-19].

IGF-IR inhibition: Based on preclinical evidence suggesting a role for IGF-IR signaling in the development of trastuzumab resistance, novel IGF-IR-targeted agents have been introduced into pharmaceutical testing and are being assessed in preclinical trastuzumab-resistant models. Therapeutic strategies that target both the HER2 and IGF-I signaling pathways should be studied further for potential use in cancers that progress on trastuzumab.

PI3K inhibition: Inhibitors of pathways downstream of the HER2 receptor may combat trastuzumab resistance additionally, drug discovery programs are focusing on developing more effective, less toxic, direct inhibitors of the Akt kinase family[16].

ErbB2-specific tyrosine kinase inhibitors:

Overexpression of the ErbB2 receptor can lead to increased tyrosine kinase activities triggering down-stream signaling pathways and subsequent biological functions. Among the many inhibitors identified, emodin (3-methyl-1,6,8-trihydroxyanthraquinone) suppresses the autophosphorylation and transphosphorylation activities of ErbB2 tyrosine kinase in ErbB2-overexpressing breast cancer cells and results in suppression of tumor growth .Similarly, curcumin, a natural compound present in turmeric, was shown to inhibit the tyrosine kinase activity of p185^{ErbB2} by depleting p185^{ErbB2} and it inhibited growth of p185^{ErbB2}-overexpressing breast cancer cell lines [15-17].

ErbB2 and Trastuzumab: Details on Clinical efficiency and resistance

Trastuzumab is active as a single agent and in combination with chemotherapy in HER2overexpressing MBC, leading to FDA approval of Trastuzumab in 1998 for treatment in this setting. The objective response rates to Trastuzumab monotherapy were low, ranging from 12% to 34% depending on prior therapy for metastatic disease, for a median duration of 9 months. Hence, the majority of HER2-overexpressing tumours demonstrated primary (*de novo* or intrinsic) resistance to single-agent Trastuzumab. In fact, the rate of primary resistance to single-agent Trastuzumab for HER2-overexpressing MBC is 66% to 88% [20]. Further phase III trials revealed that combining Trastuzumab with Paclitaxel [21] or Docetaxel [21-25] could increase response rates, time to disease progression, and overall survival compared with Trastuzumab monotherapy. In patients whose tumours had amplified *her2* and had not received prior chemotherapy for MBC, the median time to progression in response to single-agent Trastuzumab treatment was 4.9 months [22]; in patients who received Trastuzumab and chemotherapy, the median time to progression was 7.4 months [23]. Thus, the majority of patients who achieve an initial response to Trastuzumab-based regimens develop resistance within one year. In the adjuvant setting, administration of Trastuzumab in combination with or following chemotherapy improves the disease-free and overall survival rates in patients with early stage breast cancer [23-25]. However, approximately 15% of these women still develop metastatic disease despite Trastuzumab-based adjuvant chemotherapy. Elucidating the molecular mechanisms underlying primary or acquired (treatment-induced) Trastuzumab resistance is critical to improving the survival of MBC patients whose tumours over express HER2 [22-28].

Studies on Trastuzumab resistance

Steric hindrance of receptor-antibody interaction: overexpression of MUC4

A potential mechanism by which resistance to targeted antibodies may develop is via disruption of the interaction between the therapeutic agent and the target protein. Resistance to Trastuzumab was associated with increased expression of the membrane-associated glycoprotein MUC4 [26]. MUC4 was shown to bind and satirically hinder HER2 from binding to Trastuzumab [26,27]. MUC4 has been suggested to contribute to cancer because of its ability to inhibit immune recognition of cancer cells, promote tumour progression and metastasis, suppress apoptosis, and activate HER2 [28]. MUC4 interacts directly with HER2, an event that is dependent upon an epidermal growth factor (EGF)-like domain on the ASGP-2 subunit of MUC4 [26]. Through this interaction, it is proposed that MUC4 serves as a ligand for HER2, resulting in increased phosphorylation of HER2 on the residue Tyr1248 [26], which is a major phosphorylation site contributing to the transforming ability of the HER2

oncoprotein [29]. MUC4 does not affect total HER2 receptor expression levels [26,28]. The JIMT-1 Trastuzumab-resistant cell line described by Nagy and colleagues [28] was established from a breast cancer patient showing *her2* gene amplification and primary resistance to Trastuzumab [30]. Using this model, the authors demonstrated that the level of MUC4 protein was inversely correlated with the Trastuzumab binding capacity, and showed that knockdown of MUC4 increased the sensitivity of JIMT-1 cells to Trastuzumab [27]. Thus, the authors proposed that elevated MUC4 expression masks the Trastuzumab binding epitopes of HER2, resulting in steric hindrance of the interaction between this antibody and its therapeutic target, resulting in drug resistance. Interestingly, the authors also reported that HER2 was unable to interact with other proteins, such as EGFR or HER3, because of epitope masking by MUC4.

Insulin-like growth factor-I receptor signaling

Trastuzumab resistance has been associated with increased signaling from the insulin-like growth factor-I receptor (IGF-IR). Increased expression of IGF-IR was shown to reduce Trastuzumab-mediated growth arrest of HER2-overexpressing breast cancer cells [31]. Expression of IGF-binding protein 3, which blocks IGF-I-mediated activation of IGF-IR, restored Trastuzumab sensitivity. Inhibition of IGF-IR signaling, either by antibody blockade or IGF-IR tyrosine kinase inhibition, restored Trastuzumab sensitivity in our *in vitro* resistant model, demonstrating the potential importance of this pathway as a therapeutic target in Trastuzumab-resistant breast cancer. Similar to Lu and colleagues [32], down regulation of p27^{kip1} upon IGF-I stimulation was observed in both parental and resistant cells [33]. Importantly, antisense oligonucleotides [34] and small interfering RNA [35] that reduced p27^{kip1}expression levels also blocked Trastuzumab-mediated growth arrest in HER2-overexpressing SKBR3 breast cancer cells. Transfection of p27^{kip1}or pharmacological

induction of p27^{kip1} by the proteasome inhibitor MG132 restored Trastuzumab sensitivity in our resistant model [36]. These results suggest that p27^{kip1} is a critical mediator of Trastuzumab response, and that its down regulation may occur subsequent to increased signaling from growth factor receptors such as IGF-IR, promoting resistance to Trastuzumab.

PTEN and PI3K signaling

Growth factor receptor tyrosine kinases, such as HER2 and IGF-IR, activate the PI3K signaling pathway. Constitutive PI3K/Akt activity was previously shown to inhibit cell-cycle arrest and apoptosis mediated by Trastuzumab [34]. Furthermore, Trastuzumab-resistant cells derived from the BT474 HER2-overexpressing breast cancer line demonstrated elevated levels of phosphorylated Akt and Akt kinase activity compared with parental cells [35]. These resistant cells also showed increased sensitivity to LY294002, a small molecule inhibitor of PI3K. Nagata and colleagues provided compelling evidence supporting a role for the PI3K/Akt pathway in Trastuzumab resistance. They demonstrated that decreased levels of the PTEN phosphatase resulted in increased PI3K/Akt phosphorylation and signaling and blocked Trastuzumab-mediated growth arrest of HER2-overexpressing breast cancer cells. Importantly, they showed that patients with PTEN-deficient HER2-overexpressing breast tumours have a much poorer response to Trastuzumab-based therapy. Furthermore, they showed that, in PTEN-deficient cells, PI3K inhibitors rescued Trastuzumab resistance in vitro and in vivo. These results suggest that PTEN loss may serve as a predictor of Trastuzumab resistance, and that PI3K inhibitors should be explored as potential therapies in patients with Trastuzumabresistant tumours expressing low levels of PTEN protein.

Serum HER2 extracellular domain

The full-length 185 kDa HER2 protein has been reported to be cleaved by matrix metalloproteases into a 110 kDa extracellular domain (ECD), which is released into cell culture media [37] or circulating in serum in vivo [38], and a 95 kDa amino-terminally truncated membrane-associated fragment with increased kinase activity [39]. Elevated serum levels of HER2 ECD correlate with poor prognosis in patients with advanced breast cancer. Of potential importance, Trastuzumab blocked HER2 ECD proteolytic cleavage in vitro [37-38], and patients with elevated pre-treatment ECD levels had higher response rates to Trastuzumab. HER2 overexpression in breast cancers correlated with elevated pre-treatment levels of circulating HER2 ECD in patients treated with Trastuzumab and Paclitaxel, and among these patients, responses correlated with a decline in ECD levels over 12 weeks of therapy versus lower responses in those whose ECD levels remained high post-treatment [38]. Zabrecky and colleagues [39] first described the presence of cleaved ECD in the culture medium of HER2-overexpressing SKBR3 breast cancer cells. The authors showed that HER2-targeted monoclonal antibodies bound to circulating ECD, competing away binding to membrane-bound HER2. Hence, signaling from the receptor form of HER2 continued in the presence of HER2 antibodies, indicating that HER2 ECD promoted resistance to HER2targeted antibody therapy.

Summary

Approximately 20 - 35% of invasive breast cancers exhibit overexpression of the human epidermal growth factor receptor ErbB2 tyrosine kinase receptor. Recognizing the overexpression of the ErbB2 receptor has detrimental roles leading to increased cancer metastasis and patients' poor response to anticancer therapies. Trastuzumab, an effective monoclonal antibody of choice have been used clinically to target ErbB2 receptors. However, majority of patients with metastatic breast cancer who initially respond to Trastuzumab develop resistance within one year of treatment initiation, and in the adjuvant setting 15% of patients still relapse despite Trastuzumab-based therapy. Hence it is necessary to elucidate a rationale for developing therapeutic strategies to enhance their clinical efficacy.

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Chapter 2

Major Endocytic Routes in Mammalian Cells

Adopted Strategies to study ErbB2 endocytosis in cancer cells

Cell type dependent ErbB2 endocytosis using EC-1 Peptide: an artificial ligand for ErbB2 endocytosis in cancer cell

Endocytosis or uptake is characterized by the internalization of molecules from the cell surface into internal membrane compartments, and vesicular trafficking can be divided into two main pathways - the classic, clathrin-mediated endocytic pathway and the non-classic, clathrin-independent, but lipid-raft dependent route [1]. Protein–lipid and protein–protein interactions control the targeting of signalling molecules and their partners to various specialized membrane compartments in these pathways[1-3]. This functions to control the activity of signaling cascades and the termination of signalling events, and therefore has a key role in defining how a cell responds to its environment.

Clathrin-dependent pathway

The central defining feature of the classic clathrin-dependent endocytic pathway is the recruitment of soluble clathrin from the cytoplasm to the plasma membrane [4]. The clathrin triskelia assemble into a polygonal lattice at the plasmamembrane to form coated pits that bud and pinch off from the membrane in a dynamin-dependent manner and give rise to clathrin-coated vesicle. Clathrin-binding adaptors, such as adaptor protein-2 (AP2), bind to clathrin directly to initiate this process, and they also bind to cargo proteins and thereby mediate their endocytosis[3]. In addition, phospholipids, such as phosphatidylinositol-4,5-bisphosphate, are also found in coated pits and they facilitate vesicle formation and budding by binding to clathrin adaptors such as epsins and dynamins. Clathrin-coated vesicles are uncoated after endocytosis and then fuse with the early endosome.

Clathrin independent endocytic systems

The recent development of new techniques, reagents and markers has provided new insights into non-clathrin-mediated internalization pathways [5]. Clathrin-independent internalization routes are sensitive to cholesterol depletion, which has led to the idea that they are lipid-raft dependent- a concept that is further supported by the fact that many lipid-raft-bound components seem to be endocytosed through non-clathrin pathways. Non-clathrin pathways seem to be further subdivided between those that are dynamin-GTPase dependent (CLIC--D) and those that are dynamin-GTPase independent (CLIC DI). In contrast with clathrinmediated endocytosis, almost nothing is known about the machinery that regulates the biogenesis of vesicles in these non-classic routes [6-7]. However, the lipid-raft-resident protein caveolin might have an important role in a subset of these pathways. Non-clathrin endocytic pathways can deliver molecules to various intracellular compartments that include the Golgi apparatus and the ER as well as to classic endocytic compartments, such as the recyclingendosomes.



Fig2: Major endocytic routes in mammalian cells

Adopted Strategies to study ErbB2 endocytosis in cancer cells

ErbB2 has been identified as a useful receptor for molecular targeting. Several small peptides identified through phage display have shown to be useful in tumor targeting and as potential anti-cancer therapies. The current study details the use of one such peptide ligand EC-1, to induce ErbB2 endocytosis in cancer cells. EC-1, a circular peptide of 20 amino acids has been shown to have binding affinity towards the extra cellular domain of ErbB2 and abolishes its phosphorylation at the kinase domain. Tyrosine 1248 of ErbB2 is one of the major auto-phosphorylation sites that couples ErbB2 to the Ras-Raf-MAPK signal transduction pathway. EC-1 was shown to effectively inhibit tyrosine phosphorylation of ErbB2 on residues Y1248 and Y877 in a dose- and time-dependent manner [8]. EC-1 induced inhibition of ErbB2 phosphorylation was evident after 15 min of treatment and the magnitude of inhibition was increased further at 90 min.; thus selectively inhibits proliferation of breast cancer cells overexpressing ErbB2 [8].

Two forms of EC-1 peptide have been used in the study (a) **EC-eGFP** (EC-1 peptide fused to eGFP gene) (b) **EC-Fc** (EC-1 peptide fused to the Fc- domain of human IgG.



EC-1 peptide NH2-WTGWCLNPEESTWGFCTGSF-COOH

Figure 3: Schematic representation of EC-eGFP and EC-Fc

Cell type dependent EC-eGFP/ErbB2 endocytosis

ErbB2 endocytosis in ovarian cancer derived SKOV-3 cells and breast cancer derived SKBR-3 cells were evaluated using EC-eGFP in our previous study. Internalized Ec-eGFP was found localized intracellularly with ErbB2 and endosomal markers in SKOV-3 cells [9]. This liganddependent uptake in SKOV-3 cells was transient and correlates with autophosphorylation status of ErbB2 at Y877. By contrast, ligand dependent endocytosis in ErbB2 over expressing breast cancer derived SKBR-3 cells remained endocytosis resistant[9-10].



(T.Hashizumae et al ;2008)

Fig 4: EC-eGFP dependent ErbB2 endocytosis in SKOV-3 and SKBR-3 cells. EC-eGFP internalized via endosomes. (A) Localization of EC-eGFP and ErbB2 in SKBr3 and SKOv3 cells. The cells were incubated with EC-eGFP for 90min at 37°C. Cells were then fixed and permeabilized, treated with primary antibody, the anti-ErbB2 antibody sc08 1:100 for 1h, followed by a secondary antibody, anti-mouse Alexa 555 1:500 for 30min.Scale bar 10 µm.

Internalization resistance in breast cancer cells has been debated in various studies. Hommelgaard et al, using the breast cancer cell line SKBR3 described the preferential association of HER2 with the plasma membrane protrusions [10], making HER2 an internalization-resistant receptor. In a study with EGFR and ErbB2 chimeric receptors, it was proposed that the cytoplasmic tail of ErbB2 did not have an internalization signal or it contained an inhibitory signal for efficient clathrin-mediated endocytosis (Sorkin et al., 1993) [1]. In contrast, EM studies have suggested that ErbB2 becomes internalized by clathrin-coated vesicles like many other receptors (Austin et al., 1995; Maier et al., 1991) [11], and the predominant opinion has been that ErbB2 heterodimers are recycled from endosomes to the plasmamembrane after internalization, thereby avoiding the ubiquitin-cCbl-mediated lysosomal pathway that the EGFR homodimers follow (Lenferink et al., 1998; Klapper et al., 2000; Yarden, 2001; Citri et al., 2003). Association of ErbB2 with heat shock proteins have been shown to stabilze it at membrane level [11] and Geldanamycin an Hsp90 inhibitor destabilizes the complex and favors receptor endocytosis [12].

Understanding the key factors / molecular mechanism behind ErbB2 endocytosis resistance in breast cancer cells is thus crucial for modulating uncontrolled receptor signaling cascades.

Trastuzumab

Monoclonal antibody Trastuzumab has also been used in this study to evaluate the endocytosis functions of cell surface ErbB2. By binding to the domain 1V of ErbB2, Trastuzumab inhibits receptor heterodimerization. The efficacy of Trastuzumab may also depend upon its ability to induce an immune response. Trastuzumab promotes apoptosis in multiple breast cancer cell lines via antibody-dependent cellular cytotoxicity (ADCC) [14].

Trastuzumab has also been shown to inhibit angiogenesis, resulting in decreased micro vessel density *in vivo* and reduced endothelial cell migration *in vitro*.

Expression of pro-angiogenic factors was reduced, while expression of anti-angiogenic factors was increased in Trastuzumab-treated tumours relative to control-treated tumours *in vivo* [14]. Though the demonstration of significant benefits with Trastuzumab for the management of ErbB2 positive breast cancer has been extensively used in treatment and adjuvant settings, primary or acquired resistance to Trastuzumab has been increasingly recognized as a major obstacle in the clinical management of this disease. In addition, there are currently no conclusive biomarkers to predict Trastuzumab response in clinical practice.

To further understand the endocytic properties of ErbB2 and its involvement in the acquired resistance to Trastuzumab, we extended our study by analysing major cell surface markers expressed on ErbB2 over expressing both breast cancer derived SKBR-3 and ovarian cancer derived SKOV-3 cells. Micro array profiling for cell surface markers expressed in both SKOV-3 and SKBR-3 cells showed significantly reduced expression of caveolin-1 and caveolin-2 was found in SKBR-3 cells as that of SKOV-3 [15]. The reduced gene expression

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and the resultant absence of caveolar proteins were confirmed further by RT PCR () and immuno-staining.



Fig 5: Immunostaining for caveolin-1 and caveolin-2 in SKBR-3 and SKOV-3 cells. Presence of caveolin-1and caveolin-2 on cell surface was confirmed by using anti-Cav-1 and anti-Cav2 monoclonal antibody. Presence of Macrophage Scavenger Receptor was confirmed as a positive control. Scale bar 10 μm.

In addition, the endocytic importance of caveolin(s) in EC-eGFP uptake observed in SKOV-3 cells was confirmed by adopting the gene silencing strategies of caveolin-1 and caveolin-2, resulted an inhibited EC-eGFP/ErbB2 uptake. These results imply the importance of caveolin(s) as contributors for ErbB2 endocytosis in breast cancer cells.



Fig 6: siRNA mediated down regulation of caveolin-1 and caveolin-2 expression in SKOV-3 cells. (A) Western blot analysis of caveolin-1 protein expression in SKOV-3 cells showing, caveolin-1 reduction in siRNA transfected cell lysate (lane1) caveolin-2 expression in transfected cell lysate (lane 2) control siRNA for caveolin-1 (lane 3) and endogenous caveolin-1 expression in SKOV-3(lane 4). (B) Western blot analysis of caveolin-2 siRNA inhibited protein expression showing, caveolin-2 reduction in siRNA transfected cell lysate (lane1), caveolin-1 expression in transfected cell lysate (lane2), control siRNA for caveolin-2 (lane 3) and endogenous caveolin-2 expression in SKOV-3 (lane 4). β-Actin protein levels are monitored for equal loading between individual lanes. Inhibition of caveolin-1 and caveolin-2 by siRNA resulted reduced EC-eGFP endocytosis in SKOV-3 cells. (C-D) caveolin-1 and 2 and control siRNA transfected SKOV-3 cells were incubated with EC-eGFP at 37^oC for 90 min to monitor endocytosis. Images were taken using confocal microscope. Scale bar 20 μm.

Summary

The ErbB or HER receptor network is frequently over expressed or deregulated in breast and ovarian tumors. ErbB2 overexpression in cancer cells can be targeted with receptor specific monoclonal antibodies or peptide ligands. However, ligand induced ErbB2 endocytosis was not well characterized in a range of cancer cells. Our previous studies details EC-1 peptide induced ErbB2 internalization in ovarian cancer derived SKOV-3 cells whereas no such internalization was observed in ErbB2 over expressing breast cancer derived SKBR-3 cells. Furthermore, the importance of caveolin-1 with relation to EC-1 peptide uptake in SKOV-3 cells was confirmed by gene silencing strategies. Endocytic resistance of ErbB2 in breast cancer cells has been controversially studied by many groups; however, no such studies showed the membrane level association of ErbB2 receptors with caveolar proteins. Since receptors and signaling molecules, its' impact in ErbB2 receptor endocytosis is worth to evaluate

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CHAPTER: 3

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Identification of Caveolin-I as a Potential Causative Factor in the Generation of Trastuzumab Resistance in Breast Cancer Cells

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Abstract

The oncogenic tyrosine kinase receptor ErbB2 is a prognostic factor and target for breast cancer therapeutics. In contrast with the other ErbB receptors, ErbB2 is hardly internalized by ligand induced mechanisms, indicating a prevalent surface expression. Elevated levels of ErbB2 in tumor cells are associated with its defective endocytosis and down regulation. Here we show that caveolin-1 expression in breast cancer derived SKBR-3 cells (SKBR-3/Cav-1) facilitates ligand induced ErbB2 endocytosis using an artificial peptide ligand EC-eGFP. Similarly, stimulation with humanized anti ErbB2 antibody Trastuzumab (Herceptin) was found to be internalized and co-localized with caveolin-1 in SKBR-3/Cav-1 cells. Internalized EC-eGFP and Trastuzumab in SKBR-3/Cav-1 cells were then delivered via caveolae to the caveolin-1 containing early endosomes. Consequently, attenuated Fc receptor mediated ADCC functions were observed when exposed to Trastuzumab and EC-Fc (EC-1 peptide conjugated to Fc part of human Ig G). On the other hand, this caveolae dependent endocytic synergy was not observed in parental SKBR-3 cells. Therefore, expressing caveolin-1 in breast cancer cells may

Key words: ErbB2, Caveolin-1, Antibody dependent cell mediated cytotoxicity (ADCC), internalization, Ec-eGFP, Trastuzumab

INTRODUCTION

As a co-receptor for other ErbB's, oncogenic receptor tyrosine kinase ErbB2, which lacks a natural ligand, can transduce strong mitogenic signals for cell transformation and proliferation by its ability to heterodimerize with the EGF receptor and with ErbB3 [1–3]. Correlation between the overexpression of ErbB2 and poor clinical prognosis is well documented in breast cancer patients, which marks this protein as a potential and effective therapeutic target [4], [5]. Controlled receptor activation and down regulation of receptor signaling are crucial for intracellular processes that are engaged by ligand binding. One of the ErbB2 targeting therapy is the administration of a humanized monoclonal antibody Trastuzumab, that binds to juxta membrane domain of ErbB2 [6], [7]. Clinical trials have shown that Trastuzumab inhibits breast cancer cell proliferation by the induction of cell cycle arrest at the G1 phase of the cell cycle [8]. Trastuzumab prevents ErbB2 hetero-dimerization with other ErbB receptors [9] and prevents its proteolytic shedding by metalloproteases [10]. Furthermore, the Fcy portion of Trastuzumab is reported to elicit antibody-dependent cell mediated cytotoxicity (ADCC) [11], [12], which probably is the dominant therapeutic activity of Trastuzumab, as Trastuzumab is ineffective in eliciting anti-tumor response in mice that lack Fcy receptors on ADCC promoting effector cells [13]. Taking the range of molecular and cellular mechanisms of Trastuzumab efficacy and of resistance into consideration, it might be possible that the dominant mechanism depends upon the context of the cancer target cell. However, in most cases, the levels of the ErbB2 surface expression on tumor cells are crucial for a potent and sustained therapeutic response to Trastuzumab.

Ligand induced endocytosis of receptor tyrosine kinases is considered to be crucial for the termination of intracellular signals that are transiently generated upon the ligand-receptor binding [14], [15]. Unlike ErbB1, which is a characterized receptor that undergoes endocytic processing and endosomal transport, the endocytosis of ErbB2 has been found to be impaired in tumor cells [16–19]. Moreover, several groups have reported that overexpression of cell surface ErbB2 can transmit its endocytic resistance to ErbB1 and its dimerization partners[17–19], resulting in a subsequent increase in the expression levels of the ErbB receptor heterodimerization partners on the cell membrane. Clathrin dependent endocytosis is a well established phenomena among the ErbB family members [20-22]. However, the distribution of ErbB2 homo dimers in caveolae before ligand activation [23], [24] and its association with membrane proteins, lipid rafts, caveolins and flotillins are well documented [25-27]. These observations imply the involvement of caveolae-dependent mechanisms in ErbB2 endocytosis. Caveolin-1 is 22-24 kDa integral membrane protein that is essential for caveolae formations, which are small, flask-shaped invaginations of the plasma membrane. In the context of endocytic function, caveolin-1 stabilizes the actin cytoskeleton and regulates the endocytic machinery by interacting with other membrane proteins [28-30]. Simultaneously, caveolae are often described as a signaling platforms, since caveolins bind to a number of signaling proteins including G protein subunits, receptor and non receptor tyrosine kinases and small GTPases [31] thereby regulating their activity in caveolae. It has been demonstrated that overexpression of recombinant caveolin-1 regulates ErbB2 mediated signal transduction in vivo [32]. Although caveolin-1 has been reported to function as both a tumor suppressor and a oncogene, the cumulative effect on tumorgenesis may be dependent on tumor cell type. Deciphering the molecular mechanism(s) and physiological significances of ErbB2 endocytosis through a caveolin dependent-pathway, might well provide new insights for novel ErbB2 targeted therapies.

Until now, an endogenous ligand(s) for ErbB2 have not yet been identified. However, a phage display study has identified several small peptides that exhibit specific binding to the extracellular domain of ErbB2 [33]. These peptides inhibit the kinase activity of ErbB2 and subsequent cell proliferation. Previously, we described the EC-1 peptide, an artificial ErbB2 ligand, fused to eGFP (EC-eGFP) that efficiently induced ErbB2 endocytosis in the ovarian cancer cell derived SKOV-3, while EC-eGFP did not induce ErbB2 internalization in SKBR-3 cells [34]. Simultaneously, by DNA microarray analysis, we found that the mRNA level of caveolin-1 in SKBR-3 cells were down-regulated [35]. In this study, using Ec-eGFP and Trastuzumab, we evaluated the involvement of caveolin-1 in ligand induced ErbB2 endocytosis by utilizing parental SKBR-3 and SKBR-3/Cav-1 cells, in which the caveolin-1 gene was stably transduced.

Materials and Methods

Antibodies and other reagents

Rabbit monoclonal anti-ErbB2, anti-Caveolin-1, anti-Caveolin-2, mouse monoclonal anti-Transferrin antibody, HRP conjugated anti-mouse IgG, anti-rabbit IgG, anti rabbit Ig G labeled Alexa Fluor 350 were from Cell Signaling Technology. Anti-EEA1 and β -actin antibodies were purchased from BD Biosciences. Trastuzumab (Herceptin) was purchased from (Genentech, South San Francisco, CA). Anti rabbit Ig G labeled Alexa Fluor 555 and 488, anti-mouse Ig G labeled Alexa Flour 555 was from Invitrogen. Anti human IgG FITC was from Sigma. Protein Assay reagents were from Thermo Scientific, VA. All other reagents used in this study were purchased from Sigma-Aldrich and Invitrogen unless otherwise specified.

Cell cultures and Transfection of Caveolin-1 into SKBR-3 cells

The human breast cancer derived cell line SKBR-3 and the human ovarian cancer cell line SKOV-3 (ATCC, Manassas, VA) were grown in supplemented RPMI 1640 medium at 37°C with 10% FBS, under an atmosphere of 5% CO₂. Full length human caveolin-1 cDNA derived from SKOV-3 cells were amplified using forward primer 5'gcggccgcATGTCTGGGGG - GCAAATAC-3 and reverse primer 5'gaattcTTATATTTCTTTCTACAAGTTG-3' . RT-PCR products were digested with Not1 and EcoR1 and then ligated to the Not1/EcoR1-digested PQCXIP retroviral vector (Clontech, Mountain View, CA) to generate PQCXIP/Cav1. The GP2-293 cell line (Clontech) was used to produce the viral particles. Production of virus particles, infection of the target cell line SKBR-3 and selection of virus infected SKBR-3 cells were performed as recommended by the vendor. Cells were transfected in parallel with the PQCXIP vector as a control. Puromycin (0.8µg/l) resistant stable caveolin-1 expressing transfectants SKBR-3 cells were generated and verified using anti -caveolin-1 antibody (1:500).

Preparation of Ec-eGFP and EC-FC

EC-eGFP and EC-Fc (EC-1 peptides fused to human IgG Fc domain) proteins were prepared as previously described [34], [35] . Briefly, for EC-eGFP, synthetic oligo nucleotide coding for EC-1 peptide, -NH2-WTGWCLNPEESTWGFCTGSF-COOH, and eGFP coding DNA were cloned into an expression vector pET28b (Novagen, WI, USA). A Full length EC-eGFP expressing plasmid was then introduced into E. coli MM294 (DE3) pLysS cells. For purification, cells harboring pET28b were grown at 37°C in LB media, induced protein expression at mid-exponential phase by adding 0.4 mM IPTG, grown for an additional 16 h, harvested and sonicated by resuspending in cold PBS. After centrifugations for 15 min at 12,000 rpm, the supernatant was collected and applied to a Ni-NTA super flow column (Qiagen) pre-equilibrated with 50 mM phosphate buffer pH 8.0 and 300 mM NaCl. EC-eGFP protein was eluted with 300 mM imidazole and was dialyzed against PBS for 2 h and the purity of recombinant EC-eGFP protein was analyzed by SDS-PAGE.

To generate EC-Fc, the above expression vectors [35] were stably transfected into Chinese hamster ovary (CHO) cells in the presence of 100 μ g/ml Hygromycin B (Wako Purechemicals, Japan). Six million of the transformed cells were seeded in 500 ml of CHO-S-SFM11 (Invitrogen) at 37°C and incubated for 5 days. The cell culture medium was collected on the 5th day, centrifuged, and passed through a pre-equilibrated protein A Sepharose column. After extensive washes, the bound protein was eluted in 1 ml fractions in 0.1 M phosphate buffer of pH 2.6, and was immediately neutralized by adding 20 μ l of 2 M phosphate buffer pH 8.0. The eluted EC-Fc proteins were then passed through a PD10 column (GE Healthcare) to remove the buffer and resuspended in PBS.

siRNA Transfection

siRNAs to Cav-1 (5'-AAGCATCAACTTGCAGAAAGA-3') and Cav-2 (5'-CCGGCTCAACTCGCATCTCAA-3') (QIAGEN) were transiently transfected into SKOV3 cells using Lipofectamine LTX (Invitrogen) to knockdown the gene expression of caveolin-1 and caveolin-2 according to the manufacturer's instructions.

Cell Surface Biotinylation

Cell surface biotinylation assay, using cleavable biotin was used to determine the amount of internalized ErbB2 in transfected and wild type SKBR-3 cells. Briefly, 80-90 % confluent SKBR-3 cells cultured in 6 well plates were washed twice with Hank's balanced salt solution

(HBSS) for 10 min at 4°C. EZ-Link[™] Sulfo-NHS-SS-Biotin (Pierce, Thermo Scientific, Rockford, IL) at a concentration of 0.5 mg/ml in HBSS was added to cells and placed on a shaking table for 20 min at 4°C. This reaction was repeated twice. Biotinylation was quenched by washing the cells with HEPES buffered RPMI supplemented with 1 % BSA and 2 mM glutamine for 10 min at 4°C. Control cells prepared for 37°C and 4°C were incubated with RPMI-BSA for 1 hour. SKBR-3 cells incubated with 30µg/ml Ec-eGFP and 1 µM Trastuzumab were kept at 37°C for different time intervals. Fresh cold RPMI-BSA was added to the cells upon completion of each time point (except for the controls), and the cells were then placed on ice. A similar set of samples processed at 4°C were also kept as controls. After incubation, all the samples were washed once in cold HBSS and non internalized cell surface biotin was removed by washing twice in stripping buffer (20 mM DTT; 50 mM Tris-HCl pH 8.7; 100 mM NaCl and 2.5 mM CaCl₂) for 20 min at 4°C. Finally, cells were washed three times in cold HBSS, and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 0.1% sodium dodecyl sulphate (SDS); 1% Triton X-100; 0.5% sodium deoxycholate; 1 mM EDTA, Protease and Phosphatase inhibitor cocktail (Sigma-Aldrich), incubated for 20 mins at 4°C and sonicated twice .Cell extracts were cleared by centrifugation for 5 min at 12,000 rpm and supernatants were incubated with 20 µl of avidin-agarose (Pierce Chemical) at 4°C overnight. Beads were washed three times in RIPA buffer, suspended in Laemmlli buffer (20% glycerol, 135 mM Tris-HCl pH 6.8, 4%SDS, 10% 2-mercaptoethanol, 0.005% BPB) with 2- mercaptoethanol, heated for 5 min at 95°C to release the bounded proteins and processed for western blotting.

Western blotting

Protein samples lysed in Laemmlli sample buffer and were then boiled for 5 min. Equal quantities of protein lysates were then analyzed on SDS-PAGE and transferred to PVDF

membrane (Bio-Rad, Hercules, CA) .The membranes were blocked with 10% skim milk, incubated with primary antibody in 0.4% skim milk/TBS for 2h, washed with TBST followed by appropriate secondary antibody in 0.4% skim milk/TBS incubation for 1 h. The proteins were visualized using Western lighting plus chemiluminescence reagent (PerkinElmer) in Light-Capture II cooled CCD camera system (ATTO, Tokyo, Japan).

Immunofluorescence analysis and confocal microscopy

For confocal microscopic observation, SKBR-3 cells and caveolin-1 transfected SKBR-3 cells were grown on 18-mm cover slips. Cells were then fixed with 4% paraformadehyde for 10 min, permeabilized with 0.1% Triton X-100/PBS and blocked with 1% BSA-PBS for 30 min at RT. For caveolin-1 localization, cells were incubated with 1:500 diluted anti-caveolin-1 antibody in 0.25% BSA/PBS for 1 h at RT, followed by staining with Alexa 555 labeled anti-rabbit IgG for 30 min. After wash, the cover slips were mounted with DAPI (Vector Laboratories and observed by confocal microscope IX81 with 60x magnification lens. (Olympus, Tokyo, Japan).

Analysis of internalized ErbB2

To analyze the internalized ErbB2 that was potentially co-localized with caveolin-1, cells were incubated with 30 μ g/ml Ec-eGFP or 1 μ g/ml Trastuzumab in 1 % BSA PBS at 4°C(data not shown) and 37°C. After incubation, the cells were fixed with 4% PFA for 10min, permeabilized with 0.1 % Triton X-100/PBS and blocked with 1 % BSA-PBS for 30 min at RT. Cells were incubated with primary antibodies: anti-rabbit Caveolin-1, ErbB2 or anti-mouse EEA1 diluted in 1:500 with BSA/PBS for 1hr at 37°C followed by appropriate secondary antibody staining for 30 min at RT. Cells were imaged as described earlier.

Lactate Dehydrogenase release for ADCC assay

Caveolin-1 expressing and wild type SKBR-3 and SKOV-3 cells that had been cultured in 96well plates were incubated with 1 μ M of EC-Fc and Trastuzumab for 4 h at 37°C. Peripheral blood monocytes (PBMC) were isolated from human whole blood sample that were obtained from a healthy donor using Ficoll plus (Invitrogen) gradient centrifugation. Isolated PBMCs were washed three times with PBS and incubated together with antibody pre-treated SKBR-3 and SKOV-3 cells at an effector: target ratio of 25:1, 50:1,100:1 for 4 h at 37°C. After 4 h, 96 well plates were centrifuged at 1,300 g for 10 min. The cell supernatant was then transferred to a fresh 96 well plate to determine the amount of LDH released using colorimetric assay (LDH Assay Kit, Promega) as per the vendor's protocol. The percentage of cytotoxicity was calculated as : (experimental - effector spontaneous - target spontaneous) / (target maximum - target spontaneous) x 100, where the "experimental" corresponds to the signal measured in the presence of PBMCs alone, "target spontaneous" corresponds to the signal measured in the presence of tumor cells alone, and "target maximum" corresponds to the signal measured in presence of detergent lysed in1% TritonX-100 cells.

Statistical Analysis

The Data is expressed as the mean \pm SE. The statistical significance of differences between means was determined using Student's t test. Differences were statistically significant at P < 0.05.

RESULTS

Localization of ectopic caveolin-1 in SKBR-3 cells.

Previous work by us [34], [35] and others (Verma et al., 2010) indicated the loss of caveolin-1 gene expression and caveolae in SKBR-3 cells. In order to investigate the relevance to ErbB2 internalization, a construct constitutively expressing human full-length caveolin-1 was transfected into SKBR-3 cells. Immunofluorescence microscopy of transfected cells demonstrated caveolin-1 was frequently expressed on the cell membrane Fig. 1A, and also as small or large punctae vesicles throughout the cytoplasm Fig. S2 A, which may represent caveosomes, identified previously by Pelkmans et al. [37]. The cellular localization of these vesicles were similar to the localization pattern as reported previously in SKBR-3 (Verma et al., 2010), and that of SKOV3 Fig. S2 B, which endogenously expresses caveolin-1 [34]. Cell lysates prepared from transfected and wild type SKBR-3 cells were subjected to western blot analysis with α -caveolin-1 specific monoclonal antibody Fig.1B. A significant increase in caveolin-1 protein levels was detected in transfected cells whereas wild type SKBR-3 cells were caveolin-1 negative, consistent with our previous study [34]. SKOV-3 were used as positive controls for analysis since caveolin-1 was detected in SKOV-3 [34]. The results confirmed the expression of recombinant caveolin-1 in the transfected SKBR-3 cells (SKBR-3/Cav-1), which was equivalent with the level of endogenous caveolin-1 in SKOV-3 cells.



Fig 1. Expression of caveolin-1 in SKBR-3 cells (A) Both SKBR-3/Cav-1 and parental SKBR-3 cells were processed for immunofluorescence imaging, detecting Bright field image in (DIC) caveolin-1(red) and nuclei (blue). Scale bar 10 μ m. (B) Western blot showing caveolin 1 expression in, PQCXIP vector transfected (lane1), Caveolin-1 transfected (SKBR-3/Cav-1) (lane 2) parental SKBR-3 (lane 3), and SKOV-3 cells (lane 4). β -Actin was used as loading control.

ErbB2 internalization is enhanced in SKBR-3 cells expressing Caveolin-1.

To address whether efficient ErbB2 endocytosis is associated with caveolin-1 expression in SKBR-3 cells, we treated caveolin-1 transfected SKBR-3 cells and wild type cells with EC-eGFP, an artificial ErbB2 peptide ligand. Cell surface binding of EC-eGFP was observed together with ErbB2 within 5 minutes of incubation Fig. 2A. However, induced endocytosis and intracellular localization of EC-eGFP was observed in transfected cells after 15 minutes of incubation at 37°C Fig. 2B. In wild type SKBR-3 cells, ErbB2 was retained on the cell

surface even after 60 min of incubation with EC-eGFP at 37 °C Fig.2 C. We also analyzed the effect of Trastuzumab in caveolin-1 expressing SKBR-3 cells after ligand stimulation Fig. 2D, and after 15 minutes of incubation at 37 °C. After the addition of Trastuzumab, ErbB2 in SKBR-3/Cav-1 expressing cells was observed intracellularly Fig. 2E whereas Trastuzumab above scarcely altered the cellular membrane distribution of ErbB2 in wild type SKBR-3 cells. These results suggest that upon the binding to ErbB2, EC-eGFP and Trastuzumab are effectively internalized with ErbB2 through a caveolin-1 dependent mechanism. As a negative control for endocytosis, we incubated SKBR-3/Cav-1 cells with eGFP and analyzed the endocytosis and localization of ErbB2 and caveolin-1.



Fig 2 : Enhanced internalization of ErbB2 in caveolin-1 expressing SKBR-3 cells. (A-C) Transfected and wild type SKBR-3 cells were incubated with 1µM of EC-eGFP at $37^{\circ}C$ and intracellular co-localization was assessed using anti-ErbB2 monoclonal antibody. EC-eGFP was observed as green and ErbB2 stained red. Intracellular co-localization of ErbB2 with EC-eGFP is shown in the merged image



Fig 3: Enhanced internalization of ErbB2 in caveolin-1 expressing SKBR-3 cells. (D-F). Trastuzumab treatment stimulates ErbB2 endocytosis in transfected SKBR-3 cells: both wild type and Cav-1 expressing SKBR-3 cells were grown in 18mm cover slips, treated with 1 μ M Trastuzumab for 15 minutes and processed for endocytosis analysis as explained in methods. Cells were further stained with anti human Ig G labeled FITC (1:500), anti rabbit Alexa 350 (1:500-for caveolin-1) and visualized using confocal microscope. Scale bar 20 μ M.

We further accessed for the involvement of caveolin-1 in ligand induced ErbB2 endocytosis by biotinylation Fig.3. Surface biotinylated cells were treated with 1 μ M EC-eGFP and Trastuzumab at 37 °C for various intervals. Surface biotinylated proteins on cell surface were then removed to ensure only internalized biotinylate proteins were being assessed. Cell lysates were pull-downed with avidin agarose and blotted against anti-ErbB2 monoclonal antibody to analyze the endocytosed ErbB2 in both caveolin-1 transfected and wild type SKBR-3 cells. As for the control, the same set of samples, but the ligand or Trastuzumab incubation were carried out at 4°C, were processed simultaneously for internalization. Internalization of the transferrin receptor was also monitored as an endocytosis control. In EC-eGFP treated caveolin-1 transfectants, the amount of ErbB2 which was pulled down with avidin-beads was increased 15-30 minutes after ligand treatment Fig. 3A. This was followed by a gradual decrease within 90 minutes, presumably due to potential endosomal/lysosomal degradation. In contrast, no significantly pulled down of ErbB2 was detected in caveolin-1 transfected SKBR-3 cells which had been incubated with ligand at 4°C and in wild type SKBR-3 cells after EC-eGFP treatment Fig. 3B. Trastuzumab was also found to increase the amount of biotinylated ErbB2 gradually with time in the cytoplasm of caveolin-1 transfected SKBR-3 cells, and partially in wild type SKBR-3 cells at 60 minutes Fig. 3B.



Fig4. Ligand induced

ErbB2 internalization after EC-eGFP and Trastuzumab treatment. SKBR-3 cells were surface biotinylated to monitor internalization (**A**) Biotinylated caveolin-1 expressing SKBR-3 cells were stimulated with 1 μ M EC-eGFP and Trastuzumab for 15, 30 and 60 minutes at 37°C, 4°C or left untreated for 1hr at 37°C and 4°C. Densitometry performed using Image J software. (**B**) Wild type SKBR-3 cells were incubated with Ec-eGFP and Trastuzumab as described above for 15-60 minutes. Cell lysates prepared from treated samples were then immunoprecipitated with avidin agarose and subjected to western blotting using an anti-ErbB2 antibody. Endocytosed transferrin receptor was also monitored simultaneously as internal control. The results are expressed as the mean SD of three individual experiments. Since there was no significant internalization observed in parental SKBR-3 cells, densitometry was performed only for caveolin-1 expressing SKBR-3 cells.

Since only internalized proteins were biotinylated and could be pulled down with avidinbeads under our experimental condition, we concluded that ErbB2 was endocytosed upon the ligand/Trastuzumab binding through a caveolin-1 mediated pathway.

Moreover, studies with endosomal markers to monitor post-endocytic events revealed the fusion of caveolin-1 rich vesicles with early endosomal compartments [36]. We thus analyzed

the recruitment of EC-eGFP and Trastuzumab into early endosomes using an early endosomal marker EEA1 after EC-eGFP and Trastuzumab stimulation in caveolin-1 transfected SKBR-3 cells. Localization in endosomes was not observed in transfected cells after 1 μ M EC-eGFP or Trastuzumab treatment for initial 15 minutes at 37[°]C (data not shown). However, enhanced endocytosis and localization in early endosomes was observed in transfected cells after EC-eGFP treatment for 90 min at 37[°]C Fig. 4A. Trastuzumab also found to be localized in early endosomes after incubation for 1hr Fig. 4B. Co localization of caveolin-1 with EEA1 was observed beneath the cell membrane, and as small and large caveolin-1 positive endosomal vesicles which were similar to those reported in previous studies [37], [38]. In line with those observations, these results imply a post-endocytic intersection of a caveolin-1 mediated pathway with the classic endosomal systems.



Fig 5. Co-localization of ErbB2 ligands with EEA1 after incubation in Caveolin-1 expressing SKBR-3 cells: (A) SKBR-3 cells stimulated with Ec-eGFP for 90mins at 37 °C were fixed with 4% PFA, permeabilized in 0.1% Triton X-100, and then incubated with a anti-EEA1 and a anti-caveolin-1 antibody for 1 hour. Cells were further stained with a rabbit IgG labeled with Alexa 350 and mouse IgG labeled with alexa 555 for 30 min and visualized by confocal microscopy. (B) Recruitment of

ErbB2 to early endosomes after Trastuzumab stimulation in transfected SKBR-3 cells was also observed after 90 minutes using an anti-EEA1 and anti caveolin-1 antibody. Cells were counter stained with mouse IgG labeled Alexa 555, rabbit IgG labeled Alexa 350 and human IgG labeled FITC for 30 minutes and co-localization was observed using confocal microscope. Scale bar 20 μ m.

Attenuated MAPK phosphorylation in Caveolin-1 expressing cells

As a result of ErbB2 internalization and subsequent endosomal degradation, considerable attenuation in the downstream MAPK phosphorylation status was observed when treated with EC-eGFP and Trastuzumab in caveolin-1 expressing SKBR-3 cells and SKOV-3 cells.

MAPK phosphorylation in parental SKBR-3 cells is the resultant continued receptor signaling from ErbB2 monomers or hetero dimers, which further substantiates the lack of receptor endocytosis



Fig6. Analysis

of P-MAPK signaling in SKBR-3, SKOV-3 and SKBR-3/Cav-1 cells. SKBR-3, SKOV-3 and SKBR-3/Cav-1 cells were incubated with EC-eGFP and Trastuzumab for 5 or15 minutes and cell lysates were prepared. (A) Western blot showing P-MAPK signals in SKBR-3, SKOV-3 and SKBR-3/Cav-1 cells for 0 minutes (control/non treated) 5min and 15min. Equal loading was confirmed by total ErbB2 assessment.(B) Relative band intensity of samples in each lanes of (A) was analyzed using ImageJ software. The results of three independent experiments were graphed as mean relative band intensity.

Attenuated ADCC effect on SKBR-3 cells expressing caveolin-1

To assess if the level of ErbB2 endocytosis is significant for SKBR-3 cells to escape from the ADCC effect, we set out to evaluate the ADCC effect mediated by human peripheral blood mononuclear cells (PBMC) on Trastuzumab or EC-Fc treated caveolin-1 transfected SKBR-3 and SKOV-3 cells Fig. 5. The PBMC mediated cytotoxicity of wild type of SKBR-3 cells was taken as controls for the ADCC effect. When the effector/target cell ratio was 100:1, 50:1, 25:1, cell death was approximately 70%, 55% and 45%, respectively in the case of wild type of SKBR-3. In contrast, a significant attenuated ADCC cell killing effect was observed in caveolin-1 expressing SKBR-3 cells with a 20-35% cell death, which was almost equivalent to the ADCC effect observed in SKOV-3 cells. This was in agreement with the protein levels of caveolin-1 in both cells types Fig. 1B. The trend of ADCC effects were similarly observed with both Trastuzumab and EC-Fc, suggesting that antibody or ligand induced internalization of ErbB2 through a potential caveolin-1 dependent pathway could be significant for desensitizing cells to the ADCC-dependent cellular cytotoxicity of Trastuzumab.





Fig.5 Fc receptor mediated ADCC in SKBR-3 cells. ADCC activity mediated by Trastuzumab and EC-Fc against cell surface ErbB2 in SKBR-3, SKBR-3/Cav-1 and SKOV-3 cells was measured using human PBMC's as effector cells at an effector: target cell ratio of (A) 25:1 (B) 50:1 and (C) 100:1 , with standard LDH assay as described in methods. Data are expressed as the mean of (\pm) SD (n=3). Student's t- test (two tailed) was used to compare the ADCC response in Cav-1 SKBR-3 and SKOV-3 with parental SKBR-3. Differences were statistically significant at P < 0.05.

DISCUSSION

This is the first report to describe a positive correlation between ligand induced ErbB2 endocytosis and caveolin-1 expression in SKBR-3 cells. Upon EC-eGFP or Trastuzumab treatment, ErbB2 was internalized in caveolin-1 expressing SKBR-3 cells Fig. 2 and in SKOV-3 cells that express endogenous caveolin-1 [34]. Reciprocally, the inhibition of ErbB2 internalization by siRNA mediated depletion of caveolin-1 and 2 in SKOV-3 cells resulted in a decreased internalization of ErbB2, which further confirmed the involvement of caveolin-1 in mediating endocytosis Fig. S2. In this context, caveolae may well function as an endocytic pathway for ErbB2 internalization. On the other hand, significant differences in the ADCC response in caveolin-1 transfected and wild type SKBR-3 cells mediated by Trastuzumab and EC-Fc further substantiates a possible functional effect of ErbB2 endocytosis in Trastuzumab resistance.

The EC-1 peptide and Trastuzumab might directly crosslink cell-surface ErbB2 thereby favoring clustering of a peptide- or antibody-receptor complex to form homo-dimers. Elevated homo dimerizations of ErbB2 after Trastuzumab stimulation in SKBR-3 cells was previously reported to localize in raft-associated regions of the cell membranes (De Lorenzo et al., 2005; Nagy et al., 2002; Nagy et al., 2010)Caveolae/ caveolin structure and functions have also been shown to be raft associated [40]. In addition, flotillins, a raft-associated protein, stabilizes caveolin-1 on the membrane (Pust et al., 2012), and facilitates complex formations with ErbB2 and Hsp90 [25]. Geldanamycin, a potential Hsp90 inhibitor, disrupts this complex and triggers ErbB2 endocytosis in SKBR-3 [41],[42]. It should be indicated here that those observations were performed in wild type SKBR-3 cell. However, our RT-PCR analysis revealed low amount of endogenous caveolin-1 in SKBR3 [35] ,which might have

physiological consequences in terms of internalization defects in SKBR-3 cells. Over expression of caveolin-1 should induce de novo formation of caveolae and therefore confer stability to caveolar vesicles [43]. Collectively with our findings, it can be speculated that in presence of caveolin-1, the homo dimerizations of ErbB2 formed after Ec-eGFP/Trastuzumab treatment could facilitates membrane bound ErbB2 to internalize into caveolin-1 containing early endosomes which would develop into caveolae. Following internalization, the endocytosed molecules may well destined to the intracellular compartments for sorting and degradation. Internalized ErbB2 has been reported to co-localize with early endosomal markers after prolonged ligand stimulation [36]. This is in agreement with our observation in transfected SKBR-3 cells, where we observed the co-localization of ErbB2 with endosomal marker EEA1 after 90 mins of Ec-eGFP/Trastuzumab treatment Fig.4, implying a possible interaction between caveolin-1 mediated endocytosis with classic endosomal system through Rab5-GTPase. [37], [38]

Although the relationship between caveolin-1 and tumorogenesis has been debated [44], in breast cancer derived cells, the tumor suppressor efficacy of caveolin-1 may related to a receptor down regulation. It should be noted that a inverse relationship between caveolin-1 and ErbB2 expression has been reported in breast cancer (Park et al., 2005; Patani et al., 2012; Sagara et al., 2004). Taking our results into consideration that ligand-stimulated ErbB2 facilitates endocytic uptake via caveolae, thus caveolin-1 should contribute to inhibit growth/proliferation signals from ErbB2, thereby functioning as a tumor suppressor [48-49]. On the other hand, caveolin-1 and caveolae deficiency might be preferable in the clinical administration of Trastuzumab. As a consequence of enhanced endocytosis, we observed attenuation in Fc receptor PBMC activity mediated by Trastuzumab and EC- Fc in caveolin-1 expressing SKBR-3 cells and SKOV-3 cells. An effector /target ratio dependent ADCC

response was observed markedly in wild type SKBR-3 cells after 4hrs of incubation with EC-Fc /Trastuzumab Fig. 5. ErbB2 was retained on the cell surface even after ligand stimulation in caveole deficient breast cancer cells. The attenuated ADCC effect caused by the antibody mediated endocytosis of ErbB2 by caveolin-1 might be one mechanism that could contribute to Trastuzumab resistance. Thus, collectively, we propose caveolin-1 expressed SKBR-3 cells as another model to explain Trastuzumab resistance in ErbB2 overexpressing cell lines. The expression levels of caveolin-1 might be a predictive marker to distinguish tumor cells that respond favorably to Trastuzumab from Trastuzumab resistant ones.

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Conflict of interest

The authors declare no conflict of interest

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