Biochimica et Biophysica Acta 1862 (2016) 601–610

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Altered S-nitrosothiol homeostasis provides a survival advantage to breast cancer cells in HER2 tumors and reduces their sensitivity to trastuzumab

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 21 January 2016
Accepted 4 February 2016
Available online 6 February 2016

Keywords:
Apoptosis
Breast cancer
S-nitrosoglutathione reductase
HER2/neu
Nitric oxide
S-nitrosylation

ABSTRACT

The monoclonal antibody trastuzumab against HER2/neu, which is overexpressed in 15–20% of breast cancers, has clinical efficacy but many patients do not respond to initial treatment or develop resistance during treatment. Nitric oxide (NO) regulates cell signaling by targeting specific cysteine residues in proteins, forming S-nitrosothiols (SNO) in a process known as S-nitrosylation. We previously reported that molecular characteristics in breast cancer may dictate the tumor response to impaired SNO homeostasis. In the present study, we explored the role of SNO homeostasis in HER2 breast tumors.

The antiproliferative action of trastuzumab in HER2-overexpressing BT-474 and SKBR-3 cells was suppressed when S-nitrosoglutathione reductase (GSNOR/ADH5) activity, which plays a key role in SNO homeostasis, was specifically inhibited with the pyrrole derivative compound N6022. Moreover, GSNOR inhibition restored the activation of survival signaling pathways involved in the resistance to anti-HER2 therapies (AKT, Src and c-Abl kinases and TrkA/NRTK1, TrkB/NRTK2, EphA1 and EphA3 receptors) and reduced the apoptotic effect of trastuzumab. Accordingly, GSNOR inhibition augmented the S-nitrosylation of apoptosis-related proteins, including Apaf-1, pSer73/63 c-Jun, calcineurin subunit α and HSF1. In agreement with in vitro data, immunohistochemical analyses of 51 breast tumors showed that HER2 expression was associated with lower expression of GSNOR protein. Moreover, gene expression analysis confirmed that high ADH5/GSNOR gene expression was associated with high patient survival rates in HER2 tumors.

In conclusion, our data provide evidence of molecular mechanisms contributing to the progression of HER2+ breast cancers and could facilitate the development of therapeutic options to counteract resistance to anti-HER2 therapies.

1. Introduction

Breast cancer is the most common malignancy in women and one of the leading causes of death among women. In 2014, the rate of incidence of breast cancer in the United States represented 29% of all cancer cases with 15% of total cancer deaths that year [43]. In Europe, the rate of incidence of breast cancer was 29%, with an estimated 17% of deaths from this tumor. About 15–20% of patients with breast cancer have HER2/neu (human epidermal growth factor receptor 2) positive tumors, and overexpression or amplification of HER2 has been shown to be an important predictor for both overall survival and for the time to relapse in these patients [31]. The use of the humanized monoclonal antibody trastuzumab as therapy directed against HER2 has shown considerable clinical efficacy and increased overall survival of patients

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http://dx.doi.org/10.1016/j.bbadis.2016.02.005
0925-4439/

with HER2 positive breast tumors [3]. Trastuzumab has been shown to induce tumor regression in about a third of patients with HER2-positive metastatic cancer, but this response is hardly sustainable only with trastuzumab as monotherapy [5]. Moreover, the overall response rate to trastuzumab remains modest since about 26% of patients respond when trastuzumab is administered as monotherapy, and 40–60% of patients will respond when used in combination with systemic chemotherapy [41,51]. Many patients do not respond to initial treatment with trastuzumab (de novo resistance), and many others develop resistance to trastuzumab after continued treatment (acquired resistance) [38,51]. Also, about 10% of patients develop distant recurrence after trastuzumab-based adjuvant chemotherapy, and all patients with metastatic breast cancer eventually develop disease progression. The causes of these failures to anti-HER2 treatment are not well known and more research is needed on those molecular mechanisms involved in HER2 signaling and their response to targeted therapies.

In recent years a number of studies have provided evidence that nitric oxide (NO) may regulate cell signaling by modifying target proteins through reaction with a thiol group in specific cysteine residues, forming a S-nitrosothiol (SNO) in a process commonly known as S-nitrosylation [29]. Several enzymatic mechanisms play important roles in SNO metabolism and therefore in the regulation of this post-translational modification [28,29]. One such factor is nitrosoglutathione reductase (GSNOR), a highly evolutionarily conserved enzyme that plays a key role in SNO homeostasis [26]. Altered SNO metabolism and S-nitrosylation of proteins play a key role in pathologies such as arthritis, diabetes, multiple sclerosis, asthma, cystic fibrosis, pre-eclampsia, and septic shock [17]. However, there are scarce studies addressing the participation of SNO metabolism in cancer.

We have previously shown that molecular characteristics in breast cancer may dictate the tumor response to impaired SNO homeostasis, and that the molecular mechanisms involved may also play a significant role in the development of resistance against hormonal therapies [11]. Significantly, the present study shows that anti-tumoral action exerted by trastuzumab in HER2-overexpressing breast cancer cells is suppressed when GSNOR activity is inhibited. Moreover, our study supports that altered SNO homeostasis provides a survival advantage to cancer cells in HER2 + tumors and may constitute a mechanism of resistance to anti-HER2 therapy in breast cancer.

2. Material and methods

2.1. Materials

N6022 was obtained from Axon Medchem (Groningen, Netherlands). A 50 mM stock solution of N6022 was prepared in DMSO and diluted in culture medium and added to cells as described in figure legends. The final concentration of DMSO was 0.001%, which was added alone at this concentration as vehicle control. The monoclonal antibody trastuzumab (Herceptin®, Roche), was prepared in 7.2 ml distilled water at 21 mg/ml stock concentration, aliquoted and stored at −20 °C. The necessary aliquots were thawed and diluted to the appropriate concentration in culture medium.

2.2. Cell culture

MCF-7, and BT-474 cells were from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and obtained through Sigma-Aldrich (Madrid, Spain). Cells were grown in MEM with Earle's salts (PAA Laboratories GmbH, Pasching, Austria), containing 15% FBS (PAA) and supplemented with 2 mM glutamine, 1% non-essential aminoacids, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). SKBR-3 cells were from the ATCC Cell Lines Services (CLS GmbH, Eppelheim, Germany), and were cultured in DMEM (Gibco™ Dulbecco's Modified Eagle Medium, Life Technologies, Carlsbad, CA, USA), containing 10% SFB and supplemented as above.

2.3. Cell proliferation and cell death assays

Cells were seeded in 96-wells plates, treated as described in figure legends and cell proliferation was assayed using the XTT Cell Proliferation Assay Kit (Roche, Basel. Switzerland) following the manufacturer's instructions. The dye produced by viable cells was spectrophotometrically detected (450–655 nm) using an ImarkTM Microplate Reader (Biorad, Hercules, Ca, USA). In each assay, cell proliferation was expressed as percentage of untreated cells. Apoptotic cell death was measured using FITC-conjugated Annexin V/propidium iodide assay (Bender MedSystems Inc., Vienna, Austria) following the manufacturer's recommendations. Flow cytometry was performed in a FACSCalibur (BD Biosciences, San Jose, California, USA) to quantify the percentage of apoptotic cells.

2.4. Cell cycle analysis

Cells ($0.5–1 \times 10^6$ cells) were trypsinized and resuspended in PBS. Ice-cold 100% ethanol was added in a drop-wise manner while gently vortexing and incubated for 20 min at room temperature. Samples were centrifuged at $300 \times g$ for 5 min, resuspended in PBS containing 50 μg/ml propidium iodide plus 100 μg/ml RNase A and incubated for 20 min at room temperature protected from light. Analysis and measurement of propidium iodide fluorescence were performed on a FACSCalibur (BD Biosciences) flow cytometer (FACS; BD, Franklin Lakes, NJ, USA).

2.5. Immunoblotting

Cells grown in 60 mm dishes were harvested with cold PBS and after centrifugation ($1500 \times g$, 4 °C, 5 min), the cell pellet was incubated 15 min on ice with 1 ml lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 10% glycerol, 1% NP40, 0.1 M dithiothreitol (DTT), 0.1 M phenylmethylsulfonyl fluoride (PMSF), 1% *v*/*v* protease inhibitor cocktail (SERVA, Heidelberg, Germany), and 1% *v*/*v* phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)], and centrifuged at $15,000 \times g$ for 15 min at 4 °C. Cell lysates were stored at 80 °C until analysis. Total protein content of the lysates was determined by a standard Bradford assay using the reagent from Bio-Rad Laboratories (Hercules, CA). Proteins were separated on SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies dissolved in TTBS followed by incubation with secondary antibody conjugated with HRP, chemiluminescent reaction with ECL Plus Western Blotting Detection System or ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences, Little Chalfont, UK). Images were captured on a ChemiDoc XRS Imaging System (BioRad Hercules, CA, USA). Sources of antibodies were as follows: polyclonal anti-ADH5 (GSNOR) was from Origene (Rockville, MD, USA), monoclonal anti-phospho-Akt (Ser473), polyclonal anti-Akt, monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204), polyclonal anti-ERK1/2, and monoclonal anti-Cyclin D1 were from Cell Signaling (Beverly, MA, USA). Monoclonal anti-HER2, polyclonal anti-actin, and secondary antibodies conjugated with horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody array (PathScan® RTK Signaling Antibody Array Kit, Cell Signaling Technology, USA) was incubated with 75 μg of cell lysate protein following the manufacturer's instructions. After samples incubation, phosphorylated proteins were detected on the nitrocellulose membrane by chemiluminescence using ImageQuant LAS4000 equipment (GE Healthcare Life Sciences, Piscataway, NJ, USA). The relative chemiluminescence of each spot was quantified by densitometry using the Quantity One software (Biorad). Data from three independent experiments were normalized to the negative controls included in the array.