Biomedicine & Pharmacotherapy 72 (2015) 37–43

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original Article

Lapatinib enhances the cytotoxic effects of doxorubicin in MCF-7 tumorspheres by inhibiting the drug efflux function of ABC transporters

So-Young Chun, Yun-Suk Kwon, Kyung-Soo Nam **, Soyoung Kim *

Department of Pharmacology, School of Medicine and Intractable Disease Research Center, Dongguk University, 87 Dongdae-ro, Gyeongju-si, Gyeongsangbuk-do 780-350, Republic of Korea

ARTICLE INFO

Article history:
Received 9 March 2015
Accepted 20 March 2015

Keywords:
Tumorsphere
Chemoresistance
Lapatinib
MCF-7 breast cancer cells
ATP-binding cassette (ABC) drug transporters

ABSTRACT

Increasing evidences indicate that cancer stem cells are resistant to chemotherapy due to their cell quiescence and the expression of ATP-binding cassette (ABC) transporters. In this study, we utilized tumorsphere cultures to seek better strategies to overcome chemoresistance since tumorsphere cultures have been used widely for the enrichment of cancer stem cells. We found that tumorspheres generated from MCF-7 human breast cancer cells exhibited high proportions of quiescent cells and expressed MDR-1 at elevated levels, leading to resistance to 5-fluorouracil, paclitaxel, and doxorubicin. Because the expression of EGFR/HER2 was increased in MCF-7 tumorspheres, we assessed the combinational effect of the dual ErbB1/ErbB2 inhibitor, lapatinib, with doxorubicin in tumorspheres. The results showed that inhibition of EGFR/HER2 signaling by lapatinib sensitized MCF-7 tumorspheres to doxorubicin by inhibiting the expression of the ABC transporters, MDR-1 and BCRP, and thus, enhancing the intracellular accumulation of doxorubicin. These findings suggest that combinations of lapatinib and cytotoxic anticancer drugs may offer an advantage for treating the drug-resistant cancers.

1. Introduction

The development of resistance to anticancer drugs is the leading cause of treatment failure in cancer. However, the mechanisms associated with the development of chemoresistance are complex and not fully understood. Recent studies have focused on the linkage between chemoresistance and a small population of cancer cells called cancer stem cells. These cells possess the capacity to self-renew and differentiate to regenerate whole tumors, whereas most cancer cells lack this regenerative capacity [1,2]. Increasing evidences indicate that cancer stem cells are resistant to chemotherapy due to their properties expressing ATP-binding cassette (ABC) drug transporters and their quiescence [3,4]. Furthermore, the expressions of ABC drug transporters, such as, p-glycoprotein (MDR-1/ABCB1), multidrug resistance-associated proteins (MRPs), and breast cancer resistance proteins (BCRP/ABCG2), have been shown to protect cancer stem cells from chemotherapeutic agents [3,5]. Besides the expression of ABC drug transporters, cancer stem cells are expected to be inherently refractory to drugs that target rapidly dividing cells because they are generally regarded as quiescent [4,6].

Tumorsphere culture has been widely utilized as a surrogate *in vitro* model of cancer stem cells since it has been proposed that cells cultured under non-adherent culture conditions form tumorsphere that is exhibiting stem cell properties [7–10]. However, several studies have shown that sphere formation from various cancer cell lines or tissues does not always predict the enrichment of cancer stem cells. Accordingly, it appears the enrichment of cancer stem cells in tumorsphere culture is either cell-line dependent or depends on experimental variables, such as, cell density and duration of culture [11–13]. Thus, extensive characterization of tumorspheres is required before it can be concluded that tumorspheres confer stem cell properties. In our previous study, although we were unable to show that tumorspheres are

Abbreviations: Lapa, lapatinib; Dox, doxorubicin; ABC, ATP-binding cassette; MRP-1, multidrug resistance-associated protein-1; MDR-1, multidrug resistance protein-1; BCRP, breast cancer resistance protein; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PI3K, phosphoinositol-3 kinase; MAPK, mitogen-activated protein kinase; AKT, protein kinase B; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* Corresponding author. Tel.: +82 54 770 2419; fax: +82 54 770 2477.
** Corresponding author. Tel.: +82 54 770 2412; fax: +82 54 770 2477.
E-mail addresses: namks@dongguk.ac.kr (K.-S. Nam), soyoungkim@dongguk.ac.kr (S. Kim).

http://dx.doi.org/10.1016/j.biopha.2015.03.009
0753-3322/

clonally derived from stem cells, we did find that tumorsphere culture provides cell quiescence [14]. Furthermore, in another study, the accumulation of cells in G0/G1 phase was reported for tumorsphere cultured hepatoma cells as compared with the same cells in monolayer culture [15]. Since quiescence is an important mechanism of drug resistance in stem cells, we optimized tumorsphere cultures for the high-throughput screening of potential therapeutic strategies that are effective against quiescent cells.

In this study, we utilized tumorsphere cultures of MCF-7 human breast cancer cells to identify better means of overcoming chemoresistance. We analyzed the cytotoxic effects of paclitaxel, 5-fluorouracil (5-FU), and doxorubicin in a tumorsphere assay to evaluate the contribution of cell quiescence on chemoresistance. Since we found that enhanced EGFR/HER2 expression is characteristic of MCF-7 tumorspheres, we investigated whether combinatorial treatment based on lapatinib (a dual ErbB1/ErbB2 inhibitor) and cytotoxic therapeutics offers a potential means of overcoming the chemoresistance of tumorspheres.

2. Materials and methods

2.1. Monolayer culture

The MCF-7 human breast cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and routinely maintained in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 10 μg/mL insulin (Welgene), and 1% antibiotic-antimycotic solution (Welgene).

2.2. Tumorsphere culture

The protocol used for tumorsphere culture was as previously described [7,13]. Briefly, MCF-7 cells were suspended in serum-free DMEM/F12 (Welgene) supplemented with 1:50 B27 (Gibco BRL, Grand Island, NY, USA), 10 μg/mL insulin (Welgene), 20 ng/mL recombinant human epidermal growth factor (EGF; R&D systems, Minneapolis, MN, USA), 10 ng/mL recombinant human fibroblast growth factor (FGF; R&D systems), and 1% antibiotic-antimycotic solution (Welgene), and cultured in non-adherent plates.

2.3. Cell kinetic assay

To examine cell proliferation rates, MCF-7 cells were plated at different concentrations (3000–20,000 cells/well) into 96 well plates under tumorspheres (see above) or monolayer culture conditions. After 4 days, premixed cell proliferation reagent WST-8 (DojinDo Laboratories, Kumamoto, Japan) was added to each well and cell viabilities were determined by measuring absorbance at 450 nm according to the manufacturer's instructions.

2.4. Cytotoxicity assay

MCF-7 cells cultured as monolayers or tumorspheres were treated with doxorubicin (Sigma, St. Louis, MO, USA), 5-fluorouracil (Sigma), paclitaxel (Sigma), lapatinib (a dual ErbB1/ErbB2 inhibitor; LC Laboratories, Woburn, MA, USA), U0126 (a MEK1/2 inhibitor; LC Laboratories), or LY294002 (a PI3K/AKT inhibitor; LC Laboratories) for 3 days and cell viabilities were measured with WST-8 reagent (DojinDo Laboratories). To examine the effects of lapatinib and doxorubicin in combination, cells were treated with doxorubicin (0.2–1 μM) in the presence of 5 μM lapatinib.

2.5. Cell cycle analysis by flow cytometry

For cell cycle analysis, cells grown as monolayers or tumorspheres for 4 days were trypsinized and fixed in cold 70% ethanol. After centrifugation, cells were washed with PBS containing 2% FBS and stained with 20 μg/mL propidium iodide (Sigma) and 200 μg/mL RNase A (Sigma) for 30 min at room temperature. The DNA contents labeled with propidium iodide were analyzed by FACSCalibur II flow cytometry (Becton Dickinson Biosciences, San Jose, CA, USA).

2.6. Intracellular doxorubicin accumulation

MCF-7 cells were cultured as monolayers or tumorspheres for 4 days, then treated with 0.5 μM doxorubicin for 1 h, trypsinized, washed twice with PBS containing 2% FBS, and resuspended in PBS containing 2% FBS. Intracellular doxorubicin was measured by its fluorescence intensity using FACSCalibur II flow cytometry (Becton Dickinson Biosciences).

2.7. Measurement of intracellular ROS production

Cells were treated with 0.4 μM doxorubicin with or without 5 μM lapatinib for 3 days, trypsinized, and incubated with 20 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma) for 30 min at 37 °C in the dark. After incubation, cells were immediately washed and resuspended in PBS containing 2% FBS. Intracellular ROS production was assessed by FACSCalibur II flow cytometry (Becton Dickinson Biosciences) by measuring the fluorescent intensity of DCF at 530 nm.

2.8. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Cells cultured as monolayers or tumorspheres were treated with 0.4 μM doxorubicin or 5 μM lapatinib for 3 days and then harvested for RNA isolation. Total RNA was extracted using the easy-BLUE™ Total RNA Extraction kit (iNtRON Biotechnology Inc., Sungnam, Korea) and cDNA was synthesized with reverse transcriptase (Takara, Shiga, Japan). RT-PCR for Cyclin D1, MDR-1, MRP-1, BCRP, and GAPDH were conducted as previously described [16]. The primer sequences used for the RT-PCR reactions were as follows:

Cyclin D1 (forward) 5′-AGCTCCTGTGCTGCGAAGTGGAAAC-3′ and
Cyclin D1 (reverse) 5′-AGTGTTCAATGAAATCGTGCGGGG-3′
MDR-1 (forward) 5′-GCCTGGCAGCTGGAAGACAAATACACAAA ATT-3′ and
MDR-1 (reverse) 5′-CAGACAGCAGCTGACAGTCCAAGAACAG-GACT-3′;
MRP-1 (forward) 5′-GCGAGTGTCTCCCTCAAA CG-3′ and
MRP-1 (reverse) 5′-TCCTCACGGTGATGCTGTTC-3′;
BCRP (forward) 5′-GCAGATGCCTTCTTCGTTATG-3′ and
BCRP (reverse) 5′-TCTTCGCCAGTACATGTTGC-3′
GAPDH (forward) 5′-ATCCCATCACCATCTTCCAG-3′ and
GAPDH (reverse) 5′-TTCTAGACGGCAGGTCAGGT-3′.

Densitometric analysis was performed using Scion Image Software (Scion Corporation, CA, USA).

2.9. Western blotting

MCF-7 cells cultured as monolayers or in suspension were lysed with RIPA buffer (50 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl pH 7.5 and 2 mM EDTA) supplemented with phosphatase and protease inhibitor cocktails (GenDEPOT, Barker, TX, USA). Lysates were centrifuged at 13,000 rpm for 20 min to remove debris, and protein concentrations were determined using bicinchoninic acid reagent (Sigma). Equal amounts of protein were separated by SDS-PAGE and