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Quercetin enhances chemotherapeutic effect of doxorubicin against human breast cancer cells while reducing toxic side effects of it

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ABSTRACT

Doxorubicin (Dox) is an efficient drug for breast cancer chemotherapy, however, its toxic side effects on non-tumor tissues, especially on myocardial cells, sometimes limit its clinical application. Therefore, it is necessary to develop a new drug, which can be combined with Dox to potentiate the anti-tumor effect of Dox at a lower concentration and attenuate the toxic side effects of it. Quercetin (Que) has anti-tumor activity in addition to its protective effects on various cells. By preparing human non-tumoral MCF-10A mammary cells, human breast cancer MCF-7 and MDA-MB-231 cells and human myocardial AC16 cells, here, we wanted to evaluate whether Que might represent such an agent and investigate its possible mechanisms of potentiating the anti-tumor effect of Dox at a lower concentration. The results showed that Que could increase intracellular accumulation of Dox in breast cancer cells through down-regulating the expression of efflux ABC transporters including P-gp, BCRP and MRP1, which can effectively eliminate cancerous cells including breast cancer stem cells (BCSCs), thereby potentiating the anti-tumor effect of Dox. Furthermore, Que attenuated the cytotoxicity of Dox to non-tumoral MCF-10A mammary cells and myocardial AC16 cells. Therefore, Que could be used as a novel agent combined with Dox in breast cancer therapy, which could potentiate the anti-tumor effect of Dox at a lower concentration and attenuate the toxic side effects of it.

1. Introduction

Breast cancer is the most common malignancy in women. Currently, surgical tumor removal combined with chemotherapy is an effective treatment for breast cancer. Doxorubicin (Dox), a kind of anthracyclines, is often classified as the first-line drugs for breast cancer chemotherapy [1,2]. Dox can cause DNA damage and induce the increased expression of tumor suppressor proteins, thus up-regulating the transcription of a number of proapoptotic genes, resulting in the death of breast cancer cells [3–5]. However, in this process, these cellular events also make Dox have toxic effects on non-tumor cells, especially on myocardial cells, and are dose-dependent to induce cell apoptosis [6,7], thus sometimes limiting the clinical application of Dox. To potentiate the anti-tumor effect of Dox at a lower concentration and attenuate the toxic side effects of it, drug combination treatment is used as a solution. An ideal drug used in combination with Dox should have anti-tumor effect as well as protective effect on non-tumor tissue. However, the drug is rarely available in clinic. Therefore, a search for such a novel drug should be at the forefront of oncology research [8].

Previous studies have shown that there are some breast cancer stem cells (BCSCs) with $CD44^+/CD24^{-/low}$ markers in breast cancer [9,10]. BCSCs are generally thought to be the source of breast cancer relapse and metastasis [11]. Hence, BCSCs should be considered to be the critical targets of doxorubicin combination chemotherapy. It is generally believed that the ATP-binding cassette (ABC) transporters are involved in the transportation of the drug, thus resulting in the change in intracellular drug concentration. Till date, 48 members of the ABC transporters family have been identified, and the ABC transporter family is divided into seven subfamilies (ABCA - ABCG) [12]. Among the 48 ABC transporters, P-gp (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2) are the most studied efflux transporters that have been shown to be closely related to the intracellular drug concentration [13]. These three transporters can transfer drugs out of cells, thus resulting in a decreased intracellular drug concentration. Therefore, if drug combination can decrease the expression of P-gp, MRP1 and BCRP, even under the premise of using a lower concentration of Dox, the intracelluar concentration of Dox will also reach the same level as before, thereby effectively eliminating cancerous cells including breast cancer stem cells. In addition to the pharmacological actions mentioned above, if the drug combined with Dox also exert beneficial effects, especially the

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protective effect on cardiovascular system, this drug may be the novel drug that be combined with Dox in the treatment of breast cancer.

Quercetin (Que) is a plant derived flavonoid present in diet. It has been shown to exert beneficial effects, including inhibiting inflammatory response [14], retarding development of arteriosclerosis [15], preventing formation of thrombosis [16], reduction mortality from coronary heart disease [17], etc. In addition, it also has been shown to exert anti-tumor effect against multiple cancer cell types, including leukemia [18], breast [19], lung [20], gastric [21], liver [22], colon [23] and prostate [24] cancer and animal models. In view of the above-mentioned pharmacological activity of Que, here, we wanted to assess whether Que combined with Dox could potentiate the anti-tumor effect of Dox at a lower concentration as well as attenuating the toxic side effects of it, and to investigate the possible mechanisms of Que in potentiating the anti-tumor effect of Dox at a lower concentration.

2. Materials and methods

2.1. Materials

Que was purchased from Sigma Inc. (USA). Doxorubicin (Dox) was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (China). Annexin V-FITC/PI Kit was purchased from 4A Biocech Co. (China). FITC-conjugated CD44 antibody and PE-conjugated CD24 antibody were purchased from eBioscience Inc. (USA). P-glycoprotein (P-gp) antibody, breast cancer resistance protein (BCRP) antibody and multidrug resistance-related protein 1 (MRP1) antibody were purchased from Santa Cruz Inc. (USA).

2.2. Cell culture

MCF-10A and MCF-7 cells, MDA-MB-231 cells and AC16 cells were respectively maintained in RPMI-1640, Leibovitz's L-15 medium and DMEM, which supplemented with 10% FBS and 1% penicillin-streptomycin.

2.3. Cell viability assay

MCF-10A, MCF-7, MDA-MB-231 and AC16 cells were respectively treated with Que, Dox, or Que combined with Dox for 24 h. MTS assay was performed according to the manufacturer's operating instructions. Firstly, the viability of MCF-7 and MDA-MB-231 cells treated with different concentrations of Que was evaluated. Since less than 0.7 μM Que had little effect on cell viability, we preferred 0.7 μM Que in the following experiments for better evaluation of its effect on the cytotoxicity of Dox. In our experiments, growth rate = (mean OD of experimental group − mean OD of blank)/(mean OD of control group − mean OD of blank) × 100%.

2.4. Cell apoptosis analyses

MCF-10A, MCF-7, MDA-MB-231 and AC16 cells were respectively treated with 2 μg/mL Dox or 2 μg/mL Dox combined with 0.7 μM Que for 24 h. Then the cells were collected and washed twice with phosphate buffered solution (PBS). The collected cells were resuspended in binding buffer at a density of 1×10^6/mL. 100 μL of the cell suspensions were incubated with 5 μL of annexin V-FITC and 10μL of PI for 15 min in the dark at room temperature. The stained cells were resuspended with PBS to 500 μL and then analysed using flow cytometry immediately.

2.5. $CD44^+/CD24^{-/low}$ phenotype detection

MCF-7 and MDA-MB-231 cells were respectively treated with 2 μg/mL Dox or 2 μg/mL Dox combined with 0.7 μM Que for 24 h. Then the cells were collected and washed twice with PBS. The collected cells were resuspended in PBS at a density of 1×10^6/mL. 100 μL of the cell suspensions were incubated with 1 μL of FITC-conjugated CD44 antibodies and 5 μL of PE-conjugated CD24 antibodies on ice for 20 min in the dark. The stained cells were resuspended with ice-cold PBS to 400 μL and then analysed using flow cytometry.

2.6. Intracellular Dox accumulation

MCF-10A, MCF-7, MDA-MB-231 and AC16 cells were respectively treated with 2 μg/mL Dox or 2 μg/mL Dox combined with 0.7 μM Que for 24 h. Then the cells were collected and washed twice with PBS. The collected cells were resuspended in PBS and the intracellular Dox accumulations were detected using flow cytometry.

2.7. Western blot analysis

MCF-10A, MCF-7, MDA-MB-231 and AC16 cells were respectively treated with 2 μg/mL Dox or 2 μg/mL Dox combined with 0.7 μM Que for 24 h. The cells were washed with pre-cold PBS twice and then collected. Total proteins were extracted with their corresponding reagents according to the manufacturer's instructions. Equal amounts of protein were respectively separated with SDS-PAGE and then transferred onto PVDF membranes. The membranes were firstly incubated with primary antibodies GAPDH, P-gp, BCRP or MRP1 overnight at 4 ℃ and then followed by second antibodies for 2 h at room temperature. Antibody detection was performed using chemiluminescence detection kit.

2.8. Statistical analysis

Each experiment was repeated at least three times. All data were presented as mean ± SD. A one-way analysis of variance was used for comparisons among the groups. Statistical analysis was performed using SPSS 13.0 software. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Que enhances cytotoxicity of DOX to tumor cells and reduces cytotoxicity of DOX to non-tumor cells

Que had little effect on the proliferation of breast cancer cells at concentrations less than 0.7 μM (Fig. 1A). Since several studies had shown that Que could inhibit the proliferation of a variety of tumor cells in a dose-dependent manner, we preferred 0.7 μM Que in our study for better evaluation of its effect on the cytotoxicity of Dox. Our results showed that nontoxic dose of Que could significantly enhance the cytotoxicity of Dox to breast cancer MCF-7 and MDA-MB-231 cells (Fig.1B and C). Meanwhile, we also evaluated the effect of Que on the cytotoxicity of Dox to non-tumoral MCF-10A mammary cells and myocardial AC16 cells. Contrary to the enhancing effect of Que on the cytotoxicity of Dox to breast cancer cells, Que reduced the cytotoxicity of Dox to normal mammary cells and myocardial cells (Fig.1D and E).

3.2. Quercetin increased tumor cell apoptosis induced by Dox and reduced non-tumor cell apoptosis induced by Dox

In order to further study the effect of Que on the cytotoxicity of Dox to different tissue derived cells, we analysed the changes in the apoptosis of those cells treated with Dox alone or Dox combined with nontoxic dose of Que using flow cytometry. The results showed that nontoxic dose of Que could synergize with Dox in inducing the apoptosis of breast cancer MCF-7 and MDA-MB-231 cells (Fig.2A and B). Contrary to the synergistic effect of Que and Dox on the apoptosis of breast cancer cells, Que reduced the apoptosis induced by Dox in non-tumoral MCF-10A mammary cells and myocardial AC16 cells (Fig.2C and D).