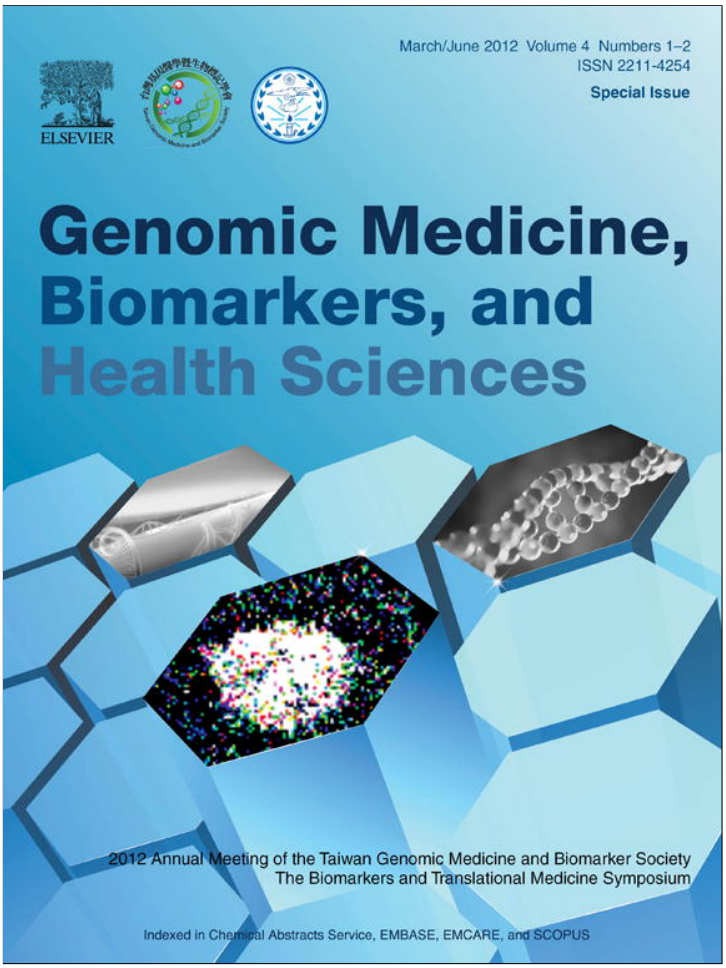

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SHORT COMMUNICATION

Proteomic analysis of quercetin-induced cardioprotective effects

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Abstract Cancer has continuously occupied the top leading cause of death in Taiwan for 29 years. Thus, scientists around the world actively devote themselves to the study of cancer therapeutics. Doxorubicin is one of the most efficient drugs in cancer therapy, but it also produces reactive oxygen species that induce serious cytotoxicity against heart cells. Quercetin, a plant-derived flavonoid, has been proven to contain potent antioxidant, antihistamine, antimicrobial, and anti-inflammatory properties. The aim of this study is to perform an *in vitro* investigation into whether quercetin is capable of decreasing doxorubicin-induced cytotoxicity and promoting the cell repair system in cardiomyocyte H9C2 cells. Proteomic analysis was performed to investigate the quercetin-induced responses.

Introduction

Doxorubicin is a chemotherapy drug commonly used in many types of cancer treatment. The main mechanism of doxorubicin is chelating DNA, inhibiting topoisomerase II, and then producing free radicals to kill cancer cells.[1] Because myocardial is particularly sensitive to reactive oxygen species (ROS), cumulative doxorubicin *in vivo* will cause irreversible damage to heart cells and restrict the clinical use of this drug.[2] To reduce the risk of developing cardiac cell damage by doxorubicin, we can "domesticate" the myocardial cells before drug treatment. Therefore, it can protect the myocardial cells from the damage caused by doxorubicin.

The aims that we will explore are the effect of quercetin to protect H9C2 cells from doxorubicin-induced cytotoxicity

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and the regulating signals and protective mechanisms of quercetin in response to doxorubicin exposure.[3] Herein, we used two-dimensional gel electrophoresis (2D-DIGE) and identified the differentially expressed proteins between untreated control cells and doxorubicin-treated cells.

Materials and methods

Cell lines, cell culture, and cell treatment

The rat cardiomyocyte cell line H9C2 was maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), l-glutamine (2 mM), streptomycin (100 μg/ml), and penicillin (100 IU/ml). All cells were incubated in a humidified incubator at 37°C with 5% CO_2. There are three groups in this study: untreated control cells, cells treated with 0.5 μM doxorubicin for 24 hours, and cells pretreated with 200 μM Quercetin for 4 hours and then incubated with 0.5 μM doxorubicin for 24 hours.

MTT cell viability assay

After specific treatment, 50 μl of MTT working solution was added to the cells in each well followed by incubation at 37°C for 4 hours. The supernatant was carefully removed. A total of 100 μl of dimethyl sulfoxide (DMSO) was added to each well and the plates were shaken for 20 minutes. The absorbance of the samples was then measured at a wavelength of 540 nm in a multiwell-plate reader. Values were normalized against the untreated samples and were averaged from four independent measurements.

Immunoblotting

Cells were washed in cold buffer solution PBS (phosphate buffered saline) and scraped in lysis buffer. Protein samples were diluted in Laemmli sample buffer and separated by one-dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS—PAGE) according to standard procedures. Gel-separated proteins were electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked for 30 minutes with 5% (w/v) bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T). Membranes were incubated overnight with primary antibody solution in TBS-T. Membranes were washed in TBS-T and then probed with peroxidase-coupled secondary antibody. After extensive washes in TBS-T, immuno-reactive proteins were visualized using the enhanced chemiluminescence method (BioVision, CA, USA).

2D-DIGE and gel image analysis

Protein sample lysed in 2D lysis buffer was labeled with either Cy3 or Cy5 for comparison on the same 2D gel. A pool of all samples was also prepared and labeled with Cy2 at the same molar ratio of Cy2/μg protein as an internal standard to be run on all gels to facilitate image matching and cross-gel statistical comparison. Labeling reactions were performed in the dark on ice for 30 minutes and then quenched with a 20-fold molar excess of free L-lysine to dye for 10 minutes. The differentially Cy3- and Cy5- labeled samples were mixed with the Cy2-labeled internal standard and reduced with DTT for 10 minutes. Carrier ampholyte IPG (immobilized pH gradient) buffer, pH 3—10, was added and the final volume was adjusted to 450 μl with 2D lysis buffer. Immobilized nonlinear pH gradient strips (pH 3—10, 24 cm) were rehydrated with the Cy-labeled samples in the dark at room temperature overnight. Isoelectric focusing was then performed using a Multiphor II apparatus (GE Healthcare, Uppsala, Sweden) for a total of 62.5 kVh at 20°C. Strips were equilibrated in 6 M urea, 30% (v/v) glycerol, 1% SDS (w/v), 100 mM Tris—HCl (pH 8.8) with 65 mM DTT for 15 minutes, and then in the same buffer containing 240 mM iodoacetamide for 15 minutes. The equilibrated IPG strips were transferred onto 24 × 20-cm 12.5% polyacrylamide gels cast between low-fluorescence glass plates. The strips were overlaid with 0.5% (w/v) low-melting-point agarose in running buffer containing bromophenol blue. The gels were run in an Ettan (GE Healthcare, Uppsala, Sweden) 12 gel tank at 4W per gel at 10°C. 2D-DIGE gels were scanned directly between the low-fluorescence glass plates using an Ettan DIGE imager. Image analysis was performed using DeCyder 2-D Differential Analysis software version 7.0 (GE Healthcare, Uppsala, Sweden) to co-detect, normalize, and quantify the protein features in the images. Features detected from nonprotein sources were filtered out. Protein spots displaying 1.5-fold average increase or decrease in abundance with a p value < 0.05 were selected for protein identification.

Protein identification by matrix-assisted laser desorption/ ionization time of flight mass spectrometer (MALDI-TOF MS)

For MALDI-TOF MS, 0.5 μl of tryptic digest was mixed with 0.5 μl of matrix solution and spotted onto an anchor chip target plate and air-dried. The peptide mass fingerprints were acquired on an Autoflex III mass spectrometer (Bruker Daltonics Inc., Bremen, Germany) in the reflector mode. Peaks were used to generate a peptide mass fingerprint, which was searched against the updated SwissProt/TrEMBL database using Mascot software version 2.2.06 (Matrix

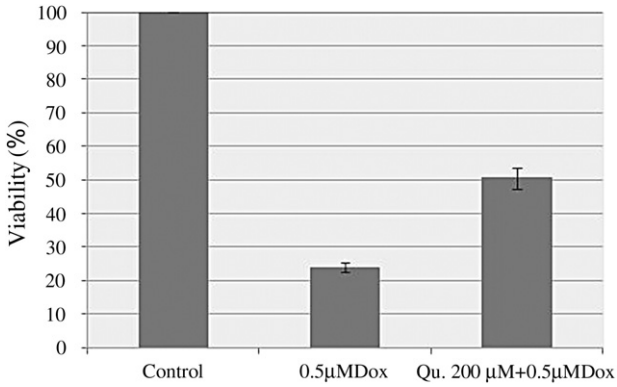

Figure 1 Effect of quercetin on the viability of doxorubicin-treated H9C2 cells. The cell viabilities were determined using MTT cell viability assay.

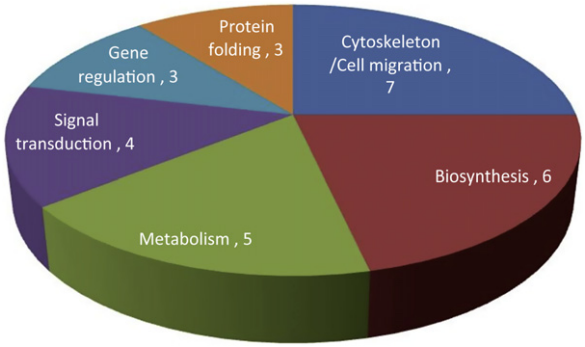

Figure 2 Functional classification of quercetin-induced changes in the protein expression of H9C2 cells.

Science, London, UK) and the following parameters: rattus, tryptic digest with a maximum of 1 missed cleavage, carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation and partial modification of glutamine to pyroglutamate, and a mass tolerance of 100 ppm.

Immunostaining and fluorescence microscopy

For immunofluorescence staining, H9C2 cells plated on coverslips and the attached cells washed twice with PBS. Localization of selected proteins was assessed using primary antibody and incubated with cells at room temperature for 1 hour. After three PBS washes, samples were incubated with appropriate fluorescently labeled secondary antibodies. For localization of filamentous actin, cells were incubated with 0.1 μg/ml Alexa 568—phalloidin (Sigma—Aldrich, OR, USA) for 45 minutes at 37°C. Coverslips were then washed three times with PBS and at least twice with ddH_2O before being mounted in Vectashield (Vector Laboratories, CA, USA) mounting medium. Coverslip edges were sealed with nail polish onto glass slides and dried in the dark at 4°C. For image analysis, cells were imaged using a fluorescence microscope.

Results

Our data demonstrated that quercetin was able to rescue the viability of doxorubicin-treated H9C2 cells (Fig. 1). However, quercetin can increase the toxic effect of doxorubicin on liver cancer HepG2 cells (data not shown). 2D-DIGE and MALDI-TOF analysis showed that quercetin regulated the expression of proteins related to cell migration, biosynthetic pathway, metabolic pathway, signal transduction, gene regulation, and protein folding (Fig. 2). To the best of our knowledge, this study is the first to demonstrate the ability of quercetin to protect cardiomyocytes from the cytotoxicity of doxorubicin and also further provide the cellular mechanism to elucidate quercetin-induced cardiomyocytes response via proteomic analysis.

Discussion

Our preliminary data show that quercetin reduces but enhances the cytotoxicity of doxorubicin on cardiomyocytes H9C2 cells and liver cancer HepG2 cells, respectively. This observation suggests that a combination of quercetin and doxorubicin on liver cancer treatment might be a helpful option for the clinical liver cancer trial.

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