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Autophagy upregulation promotes survival and attenuates doxorubicin-induced cardiotoxicity

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ABSTRACT

This study evaluated whether the manipulation of autophagy could attenuate the cardiotoxic effects of doxorubicin (DXR) in vitro as well as in a tumour-bearing mouse model of acute doxorubicin-induced cardiotoxicity. We examined the effect of an increase or inhibition of autophagy in combination with DXR on apoptosis, reactive oxygen species (ROS) production and mitochondrial function. H9C2 rat cardiac myoblasts were pre-treated with bafilomycin A1 (autophagy inhibitor, 10 nM) or rapamycin (autophagy inducer, 50 μM) followed by DXR treatment (3 μM). The augmentation of autophagy with rapamycin in the presence of DXR substantially ameliorated the detrimental effects induced by DXR. This combination treatment demonstrated improved cell viability, decreased apoptosis and ROS production and enhanced mitochondrial function. To corroborate these findings, GFP-LC3 mice were inoculated with a mouse breast cancer cell line (EO771). Following the appearance of tumours, animals were either treated with one injection of rapamycin (4 mg/kg) followed by two injections of DXR (10 mg/kg). Mice were then sacrificed and their hearts rapidly excised and utilized for biochemical and histological analyses. The combination treatment, rather than the combinants alone, conferred a cardioprotective effect. These hearts expressed down-regulation of the pro-apoptotic protein caspase-3 and cardiomyocyte cross-sectional area was preserved. These results strongly indicate that the co-treatment strategy with rapamycin can attenuate the cardiotoxic effects of DXR in a tumour-bearing mouse model.

1. Introduction

The use of doxorubicin (DXR, Adriamycin®), a broad spectrum chemotherapeutic agent, in oncologic practice has been limited by its dose-dependent cumulative cardiotoxicity, which leads to irreversible and often fatal drug-induced congestive heart failure [1–5]. The prevailing mechanism by which DXR induces cardiotoxicity is oxidative stress associated with mitochondrial dysfunction [6,7]. Although the "oxidative stress hypothesis" is supported by the ability of several antioxidants to reduce DXR-induced cardiotoxicity in animal models [8–10], these results are not reproducible in clinical trials [11]. It is therefore suggested that other mechanisms than oxidative stress might also contribute to DXR-induced heart failure. Maintenance of the structure and function of the sarcomere is essential for the protection against cytotoxicity. To ensure sufficient function of the sarcomere, precise control of protein synthesis, processing and degradation is required. Two important protein degradation systems within the heart include macroautophagy (hereafter referred to as autophagy) and the ubiquitin proteasome pathway (UPP). Autophagy is the major pathway for degradation and recycling of long-lived proteins and organelles that are sequestered in double membrane vesicles known as autophagosomes [12]. Following fusion with lysosomes to form autophagolysosomes, the inner membrane and its contents are degraded and recycled. The UPP on the other hand, functions by targeting specific proteins, labelling them with multiple ubiquitin molecules which then allow for recognition and subsequent degradation by the 26S proteasome [13,14]. It has since been appreciated that there is cross-talk between the two pathways [15] and together, these pathways play an essential role in the maintenance of sarcomeric function in the face of DXR-induced cytotoxic stimuli.

Autophagy functions as a cytoplasmic quality control mechanism to remove protein aggregates and damaged organelles. In this respect, autophagy has been shown to play a vital role in cardiac homeostasis as the inactivation of the autophagy associated gene, ATG5, results in myocardial dysfunction [16]. Alternatively, autophagic induction during ischaemia has been shown to be cardioprotective [17–19]. However, increased autophagic activity can also be detrimental to the heart under conditions such as pressure overload [20,21]. Rapamycin, a conventional inducer of autophagy through its ability to inhibit mTOR (mammalian target of

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rapamycin) [22], is a very versatile drug with well-documented effects in reducing growth in numerous cancers [23,24]. It is also being utilized as undercoats for drug-eluting stents to prevent the progression of restenosis after coronary angioplasty [25] and has been shown to be a potent inhibitor of left ventricular (LV) hypertrophy in vivo [26,27]. Although rapamycin treatment is known to be beneficial in many contexts, its potential cardioprotective effects in DXR-induced cardiotoxicity necessitate further investigation. Therefore, in this study, rapamycin pre-treatment was used as an adjuvant to ameliorate the detrimental effects of DXR such as apoptosis, ROS production and mitochondrial dysfunction.

Moreover, animal models have been widely utilized to study the molecular basis underlying DXR-induced cardiotoxicity and to develop strategies to facilitate cardioprotection. However, most of the animal models utilized only evaluated the cardiotoxic effects of DXR, without considering the effects of a growing tumour in the animal. We established a novel mammary tumour model in GFP-LC3 transgenic mice in which the effects of DXR with adjuvant rapamycin treatment could be analyzed with respect to cardiac toxicity and simultaneously assessing its effects on tumour size. The in vivo model was used to validate the potential therapeutic application of elevated autophagy in a novel tumour bearing mouse model of acute DXR-induced cardiotoxicity.

2. Materials and methods

2.1. Cell culture preparation

H9C2 rat heart myoblasts (European Collection of Cell Cultures, Salisbury, UK) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) with glutamine (Life Technologies, 32430, Johannesburg, South Africa) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Life Technologies, 10270, Johannesburg, South Africa) and 1% penicillin/streptomycin (Sigma, P4333, Johannesburg, South Africa). Incubation conditions were 100% humidified atmosphere at 37 °C in the presence of 5% CO_2. Seeding density was as follows: 1×10^6 myoblasts per 75 cm^2 tissue culture flask, 3×10^5 myoblasts per 25 cm^2 tissue culture flask, 1×10^5 myoblasts per culture dish in six-well plates and 2×10^4 myoblasts per 8-chamber slide.

2.2. Treatment of cells with rapamycin, bafilomycin A1 and DXR

At 70–80% confluency, cells were either treated with 10 nM bafilomycin A1 (Sigma, 1793, Johannesburg, South Africa) or 50 nM rapamycin (Sigma, R8781, Johannesburg, South Africa) for 6 and 24 h respectively, followed by treatment with 3 μM DXR (Sigma, D1515, Johannesburg, South Africa) for an additional 24 h.

2.3. Cell viability (MTT Assay) assessment

H9C2 myoblast viability was analyzed 24 h post treatment via the MTT (Sigma, MG2128, Johannesburg, South Africa) assay previously described by Gomez et al. [28]. At the end of the treatment protocol, 500 μl MTT (0.01 g/ml) solution was added to the cells and incubated at 37 °C for 2 h. Cell viability was quantified as the optical density (OD) determined spectrophotometrically at a wavelength of 540 nm.

2.4. Quantification of apoptosis

Caspase activity was measured using the Caspase-Glo assay (Promega, G8091, Cape Town, South Africa) following the manufacturer's instructions. This luminescent cell-based assay measures the activities of caspase-3 and -7. Following caspase cleavage, a substrate for luciferase (amino-luceferin) is released, resulting in the luciferase reaction and the production of light. Luminescence was measured using a luminometer (GLOMAX 96 microplate Luminometer, Promega) with parameters of 1 min lag time and 0.5 s/well-read time.

All assays contained appropriate controls, were performed in triplicate and repeated on three separately initiated cultures.

2.5. Preparation of cell extracts

Cell extracts were prepared as follows: lysis buffer (pH 7.4) containing (in mM): Tris–HCl 50, NP-40 1%, Na-Deoxycholate 0.25%, EDTA 1, NaF 1, SBTI 4 μg/ml, PMSF 1, Benzamidine 1, leupeptin 1 μg/ml and Triton X-100 1000 μl was added to the cells and the cell solution transferred to new tubes. Cells were sonicated, centrifuged (8000 rpm at 4 °C for 10 min), and the supernatant was transferred to new tubes. Protein concentrations were determined with the Bradford method [29], while others were diluted in Laemmli buffer and stored for Western blot analysis.

2.6. Western blot analysis

Proteins extracted from H9C2 myoblasts were subjected to either 10% or 16% gel electrophoresis to separate proteins. These proteins were transferred to nitrocellulose membranes (Immobilon™ P, Millipore, IPVH00010, Billerica, MA) which were then blocked for 2 h in Tris-buffered saline and Tween-20 (TBST, pH 7.6) containing 5% non-fat dry milk and incubated overnight at 4 °C with antibodies against LC-3 (Cell Signalling, 2775, Danvers, MA), cleaved-caspase-3 (Cell Signalling, 9665, Danvers, MA), cleaved-PARP (Cell Signalling, 9541, Danvers, MA) and anti-p62 (American Research Products, 03-GP62-C, Waltham, MA). β-Actin (Cell Signalling, 4967, Danvers, MA) was used for standardization of protein loading. The membranes were probed with appropriate secondary antibodies at room temperature for 1 h followed by washes with TBST. The protein bands were detected by chemiluminescence (ECL™, Amersham Life Science, NA934V, Johannesburg, South Africa) and quantified using densitomery (UN-SCAN-IT, Silkscience version 5.1).

2.7. Live cell imaging with fluorescence microscopy

To establish a dynamic approach of monitoring various changes within the cell and its organelles after treatment, live cell imaging was performed. For that purpose, H9C2 cells were maintained at 37 °C in growth medium and seeded in 8-chamber dishes with a density of 2×10^4 cells. Image acquisition was performed on an Olympus Cell^R system attached to an IX 81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). Using a Xenon-Arc burner (Olympus Biosystems GMBH) as a light source, images were acquired using the 360–497 or 572 nm excitation filters. Emission was collected using a UBG triple-bandpass emission filter cube (Chroma). Images were acquired through z-stacks where appropriate, using an Olympus Plan Apo N60x/1.4 oil objective. The top and bottom focus position parameter were selected, indicating the upper and lower dimensions of the sample to be acquired with a step width of 0.26 μm between the single image frames. Images were processed and background-subtracted using the Cell^R software, and presented in maximum intensity projection.

2.8. Flow cytometric analysis

Intracellular and mitochondrial ROS generation were evaluated with the aid of 6-carboxy-2′7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl ester (DCF, Molecular Probes, D399, Johannesburg, South Africa) and MitoSOX (Molecular probes, M7514, Johannesburg, South Africa) respectively. Following