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Curcumin Attenuates Doxorubicin-Induced Cardiotoxicity by Inducing Autophagy via the Regulation of JNK Phosphorylation

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Abstract

Background and Objective: Doxorubicin (DOX) has been used in cancer therapy for several decades. However, cardiac complications induced by DOX dose-dependently limit the clinical implication at optimal antitumor efficacy. Curcumin (Cur), a natural compound, has been effective as an anticancer agent in various types of cancers. It also protects against cardiac hypertrophy and heart failure, though its effect on cardiomyopathy caused by DOX treatment is unclear. To elucidate the role of curcumin on heart failure, we used the DOX induced cardiomyopathy model and primary cultured cardiac myocytes.

Method and Results: Male C57/BL6 mice were randomized to 4 courses of treatment administered for 4 weeks: Phosphate-Buffered Saline (PBS), Cur, DOX, and DOX+Cur. DOX-treated mice exhibited severe cardiac dysfunction, and the mortality was higher than that in PBS-treated mice. In DOX-treated mice, the number of apoptotic cardiac myocytes was higher and the fibrotic areas were larger than in PBS-treated mice. The cardiotoxic effects of DOX were ameliorated by treatment with Cur. In DOX-treated GFP-LC3 transgenic mice, Cur induced autophagy and decreased apoptosis in the heart. Cur also induced autophagy and suppressed DOX-induced apoptosis in neonatal rat cardiac myocytes. Inhibition of autophagy by 3-methyladenine decreased the cardioprotective effect of Cur. Furthermore, Cur decreased c-Jun N-terminal kinase (JNK) phosphorylation, resulting in reduction of apoptosis. The JNK inhibitor SP600125 abolished these effects.

Conclusion: Cur protects the heart from DOX-induced cardiotoxicity by inducing autophagy and decreasing cardiomyocyte apoptosis. The mechanism involves JNK-mediated modification of apoptosis. Induction of cardiac autophagy may be a novel therapeutic approach for preventing DOX-induced cardiotoxicity.

Keywords: Doxorubicin; Cardiomyopathy; Heart failure; Curcumin; Autophagy; Apoptosis; C-Jun N-Terminal kinase

Introduction

Autophagy is an evolutionally conserved process that involves the degradation and recycling of proteins and organelles. Primarily, autophagy is a survival mechanism that provides cells with energy in starvation conditions. It also provides a mechanism for eliminating defective organelles and recycling proteins.

Convergence of the processes of autophagy and cell death, which occurs through apoptosis or necrosis, has been reported [1,2]. If autophagic activity is insufficient, long-lived proteins and defective organelles accumulate, resulting in cell death. In contrast, if autophagic destruction of cytosol and organelles exceed a certain threshold, autophagic cell death occurs [3]. Although autophagy and apoptosis are independent processes, their cross-regulation has been investigated in the pathophysiology of human disease [4,5]. Bcl-2, which binds and inhibits the autophagic protein Beclin-1, is considered a key molecule in both autophagy and apoptosis [1,3,6].

Curcumin (Cur), a major component of turmeric (*Curcumina longa*), is a natural compound commonly used in curry or mustard. It has been used for thousands of years in China and India as an antiinflammatory medicinal agent. Antioxidant and anti-carcinogenic effects of Cur have also been reported [7,8]. In particular, its antioxidant activity contributes to cardioprotection in *in vivo* and *in vitro* models and attenuates cardiac hypertrophy and heart failure by inhibiting p300 HAT activity in animal models [8-10]. Moreover, Cur can induce autophagy, leading to autophagic cell death in cancer cells [11]. However, the effect of Cur on autophagy in cardiomyocytes is not fully understood. Doxorubicin (DOX) is a potent anthracycline antibiotic that has been used in anticancer therapy for several decades. However, its dosedependent cardiotoxicity limits its efficacy as an antitumor treatment. Several reports have described the incidence and risk of early cardiotoxicity, characterized by reversible depression of contractile function [12]. Late cardiotoxicity is more common [12,13]. Cardiac abnormalities are observed years after DOX administration; they seem to occur independently of cardiac problems that present during initial therapy [14]. Although it is widely accepted that oxidative stress with increased generation of Reactive Oxygen Species (ROS) mediates DOX-induced cardiotoxicity, the precise mechanism remains unclear.

Recently, modulation of autophagy in heart failure has received considerable attention. Tissues from patients with end-stage heart failure show evidence of autophagy [15]. However, the role of autophagy in DOX-induced cardiomyopathy, which could be protective or detrimental, is still debated [16,17]. One study showed that autophagic

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cardiomyocyte death contributed to the pathogenesis of DOX-induced heart failure and that 3-methyladenine (3-MA), a specific inhibitor of autophagy, blocked this pathway [17].

The aim of our study was to evaluate the effect of Cur on DOXinduced cardiomyopathy. We also investigated the signal transduction pathways associated with cardiac autophagy. To our knowledge, this is the first report to demonstrate that Cur can protect cardiomyocytes from DOX-induced apoptosis through the induction of autophagy.

Materials and Methods

Materials

The following reagents were purchased: DOX (Kyowa Kirin, Japan); Cur (WAKO, Japan); 3-MA, a phosphatidylinositol 3-phosphate kinase (PI3K) inhibitor (Sigma, USA); SP600125, a selective inhibitor of c-Jun N-terminal kinase (JNK) (LC Laboratories, USA); and chloroquine (SIGMA-ALDRICH).

Animal model

This investigation complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). The protocol was approved by the Institutional Animal Research Committee of Kyoto Prefectural University of Medicine. Male 8-week-old C57/BL6 mice were divided into 4 groups: control (phosphate-buffered saline [PBS]-treated mice, n=10), DOX (DOX-treated mice, n=77), Cur (Cur-treated mice, n=10), and DOX+Cur (DOX- and Cur-treated mice, n=37). DOX (20 mg/kg) was administered via a single intraperitoneal injection [18]. Since the absorption and pharmacokinetics of Cur in mice were unclear, we investigated the effect of Cur on DOX-induced heart failure as a preliminary study. We had administered Cur to the mice 5 days before DOX, 2 days before DOX and simultaneous treatment with DOX. We found that the administration of Cur intraperitoneally 5 days prior to DOX administration was most effective. Then, we decided to start Cur (1 mg/kg/day) 5 days prior to DOX successively until the day of sacrifice. PBS with 1% DMSO was administered to mice in the control and DOX groups. The GFP-LC3#53 mouse strain (RBRC00806), provided by RIKEN BioResource Center through the National Bio-Resource Project of the MEXT, Japan, was used to monitor autophagy through the fluorescence of GFP-LC3 [19].

Echocardiography and systemic hemodynamic parameters

During the 28 day observation period, body weight, heart rate, and blood pressure were monitored. Cardiac function was evaluated by echocardiography on days 4, 7, 14 and 28, as previously described [20]. One percent isoflurane inhalation was administered for sedation. Echocardiographic analysis using commercially available echocardiograph (SONOS 5500, Hewlett-Packard) equipped with a 15 MHz linear array ultrasound transducer. After the physiological studies, mice were euthanized to measure the heart weight-to-body weight ratio (HW/BW; mg/g).

Morphological analysis

Heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin or Masson trichrome staining. The same samples were used for terminal deoxynucleotidyl transferase-mediated nick-end labeling of fragmented nuclei (TUNEL) assays. For immunohistochemical analysis of cleaved poly (ADP- ribose) polymerase (PARP) and the evaluation of autophagy in GFP-LC3 mice, the heart was immediately fixed in optimum cutting temperature (O.C.T.) compound (Sakura, Japan), cryosectioned, and analyzed by microscopy.

Cardiomyocyte isolations and experimental treatment

Primary cultures of neonatal rat cardiac myocytes were prepared as described previously [21]. Cells were divided into 4 groups: a Control group in normal medium, a DOX group in medium with 1 μ M DOX, a Cur group in medium with 5 μ M Cur, and a DOX+Cur group in medium with 1 μ M DOX and 5 μ M Cur. Cur was dissolved in DMSO (nacalai tesque, Japan) just before the addition to the medium. In DOX+Cur group, DOX and Cur were administered simultaneously. DOX and/or Cur were added for 2 h to evaluate autophagy and for 24 h to evaluate apoptosis.

Evaluation of autophagy and apoptosis

Autophagy was evaluated by autophagosomal dot counts of GFP-LC3 mice frozen sections of heart tissue. DAPI (4,6-diamidino-2phenylindole) staining confirms location of the nuclei. The fluorescence of GFP-LC3 was observed under a confocal microscope (LSM510 NLO META; Zeiss, USA). The number of GFP-LC3 dots was counted in five independent visual fields from six independent mice.

In in vitro studies, autophagic flux was determined by incubating cells in the absence (steady-state autophagosomes) or presence of FBS. Western blotting for microtubule-associated protein 1 light chain 3 (LC3) of protein extracts of cardiomyocyte were adopted to evaluate autophagy. Cardiomyocytes were infected with 1 multiplicity of infection (MOI) of GFP-LC3-expressing adenovirus or LacZ virus for 48 h [22]. The TUNEL assay for the detection of apoptosis was performed according to the manufacturer's instructions (Roche, Schweiz). Nuclei were stained with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Vector Laboratories, USA). Antibodies that recognize cleaved PARP were used for immunohistochemical staining. Immunoblot analysis for confirming apoptosis was performed with an antibody specific for PARP. Samples were mounted using Fluoromount[™] (Cosmo Bio Co. Ltd. Japan), and the fluorescence of GFP-LC3 was also observed under a confocal microscope. After determining the time course of GFP-LC3 fluorescence, we evaluated the number of GFP-LC3 dots at 2 h after drug administration. The dots were counted in five independent visual fields from six independent experiments. We assessed the number of TUNEL-positive cells at 24 h after drug administration (1 μ M DOX and/or 5 μ M Cur).

Western blotting

Specific antibodies were used to detect LC3 (nano TOOLS, Germany), PARP (total and cleaved forms), cleaved PARP (large fragment of the cleaved form), JNK, p-JNK (Cell Signaling Technology, USA), Tubulin (Thermo Scientific, Japan), β -actin (Sigma), and GAPDH (Ambion, USA).

Statistical analysis

All data have been expressed as the mean \pm SEM. When more than two groups were compared, differences among the groups were determined by one-way ANOVA followed by Bonferroni/Dunn significant difference test. To evaluate the survival rate, Kaplan–Meier and log-rank tests were used to compare each group.

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Results

Cur has a protective effect on mice treated with DOX

First, we assessed mortality and cardiac function after the administration of DOX. Within the first week, 48% of mice in the DOX group had died; 60% had died at the end of the fourth week. However, in the DOX+Cur group, the mortality was 16% from day 7 to day 28, which was significantly lower than that in the DOX group. There were no deaths in the Cur and PBS groups (Figure 1A). Echocardiographic analysis was performed on days 4, 7, 14 and 28. The mice in the DOX group showed significantly increased left ventricular chamber dimensions with decreased interventricular septum and posterior wall thickness, as well as fractional shortening (% FS; P<0.01; Figure 1B, Table 1). The HW/BW (in mg/g) of mice in the DOX group was increased significantly on day 28 (Control vs DOX, 3.78 ± 0.05 vs. 4.31 ± 0.11; DOX vs. DOX+Cur, 4.31 ± 0.11 vs. 4.01 ± 0.73, P<0.05;

Supplementary Figure 1). These results indicate that administration of Cur alleviates Dox-induced cardiotoxicity and reduces mortality in mice.

Cur reduces apoptosis, induces autophagy, and prevents DOX-induced myocardial damage

Microscopic analysis of the heart showed atrophy of the myocardium and fibrosis in the DOX group on day 28. Cur suppressed these histological changes (Figure 1C). Immunohistochemical analysis of frozen heart tissue with an antibody against cleaved PARP, a marker of cells undergoing apoptosis, showed increased cleavage of PARP positive-nuclei in the DOX group mouse hearts and decreased cleavage of PARP in the DOX+Cur group mouse hearts (Supplementary Figure 2). The number of TUNEL-positive cells increased in the DOX group, indicating an increase in apoptosis in the heart. However, administration of Cur reduced the number of apoptotic myocytes in



Figure 1: Curcumin protects C57BI /6 mice heart from doxorubicin induced heart failure. (A) Cur-treated mice group with DOX showed drastic survival benefit. Cont; n=10, survival rate 100%, Cur; n=10, 100%, DOX+Cur; n=37, 87%, DOX; n=77, 40% Survival rate of DOX group on day 7 was 52%. (B) Left, Typical time course of M-mode echocardiography of both groups measured LV function at 4, 7, 28 days after DOX injection. Cur was started 5 days prior to DOX. Right, Sequential change of % FS in each group. Each bar represents mean ± SEM. *p<0.01, (n=6 per group). (C) Microscopic pathology of both group mice heart. Hematoxylin-Eosin (HE) staining and Masson trichrome (MTC) staining of mouse myocardium illustrate cardiac fibrosis, disarray as well as atrophic change of cardiomyocyte in DOX treated mice heart on 28 days. Cur suppresses these changes in DOX-treated mice myocardium. (n=6 per group). (D) In quantification of the TUNEL positive cells count, each bar represents mean ± SEM of 3 fields. *p<0.01 (n=6 per group). (E) Microscopic photograph of cardiomyocyte of GFP-LC3 transgenic mice illustrating green GFP-LC3 dots per fields, each bar represents mean ± SEM of six fields. *p<0.01 (n=6 per group). (E) Microscopic photograph of cardiomyocyte of GFP-LC3 transgenic mice illustrating green GFP-LC3 dots per fields, each bar represents mean ± SEM of six fields. *p<0.01 (n=6 per group). (E) Microscopic photograph of cardiomyocyte of GFP-LC3 transgenic mice illustrating green GFP-LC3 dots. Nuclei are stained blue with DAPI. In quantification of the GFP-LC3 dots per fields, each bar represents mean ± SEM of six fields. *p<0.01 Cont: Control; DOX: Doxorubicin, Cur: Curcumin.

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DOX group					
	pre	day4	day7	day14	day28
LVEDD	2.95 ± 0.08	3.01 ± 0.08	2.53 ± 0.10	2.98 ± 0.13	3.40 ± 0.14
LVESD	1.64 ± 0.05	1.79 ± 0.10	1.53 ± 0.08	1.76 ± 0.05	2.15 ± 0.11
IVST	0.69 ± 0.03	0.60 ± 0.04	0.65 ± 0.02	0.65 ± 0.03	0.49 ± 0.03
PWT	0.71 ± 0.04	0.61 ± 0.04	0.62 ± 0.03	0.58 ± 0.03	0.57 ± 0.03
%FS	44.4 ± 1.7	40.8 ± 2.6	39.7 ± 1.6	40.6 ± 1.4	37.0 ± 1.5
DOX+Cur group					
	pre	day4	day7	day14	day28
LVEDD	2.97 ± 0.13	2.49 ± 0.09*	2.88 ± 0.07	2.59 ± 0.11*	2.83 ± 0.06*
LVESD	1.62 ± 0.10	1.32 ± 0.05**	1.53 ± 0.09	1.43 ± 0.07	1.48 ± 0.06**
IVST	0.72 ± 0.02	0.65 ± 0.04	0.71 ± 0.04	0.73 ± 0.04	0.65 ± 0.02*
PWT	0.80 ± 0.02	0.75 ± 0.01*	0.69 ± 0.01	0.72 ± 0.02*	0.71 ± 0.02*
%FS	45.4 ± 2.1	47.0 ± 1.4	47.2 ± 2.2	44.9 ± 2.0	47.5 ± 1.5**

 Table 1: Echocardiographic Parameters during the Study Period. Echocardiographic analysis of mice subjected to DOX or DOX and Cur injection intraperitoneally. Data

 represents mean ± SEM, n=6 per each group, LVEDD: Left Ventricular End-Diastolic Dimension, LVESD: Left Ventricular End-Systolic Dimension, IVST: Interventricular

 Septum Thickness, PWT: Posterior Wall Thickness, %FS: Fractional Shortening, *p<0.05, **p<0.01 between the two groups.</td>



Figure 2: Curcumin induces autophagy and reduces apoptosis in neonatal rat cardiomyocyte after Doxorubicin treatment. (A) Confocal photomicrograph of cardiomyocyte infected with GFP-LC3-expressing adenovirus. In quantitation of GFP-LC3 puncta formation per cell, LC3 puncta were counted in representative section of the sample. Each bar represents mean \pm SEM of six independent experiments. *p<0.01 Lac2 ; control Ad-Lac2 gene transfer. (B) Western blot analysis of LC3. Data were normalized with β -actin which serves as a loading control. Each bar represents mean \pm SEM of six independent experiments. *p<0.01 Lac2 ; control Ad-Lac2 gene transfer. (B) Western blot analysis of LC3. Data were normalized with β -actin which serves as a loading control. Each bar represents mean \pm SEM of six independent experiments. *p<0.01, #p<0.05 (n=4 per group). (C) Data shown are averages of TUNEL staining of cardiomyocyte in eight fields per condition of five time experiments. Each bar represents mean \pm SEM of six independent experiments. *p<0.01, #p<0.05 (D) Representative western blot analysis to detect cleaved PARP in neonatal rat cardiomyocyte. Data were normalized with Tubulin which serves as a loading control. Each bar represents mean \pm SEM of four independent experiments. #p<0.05 Cont: Control; DOX: Doxorubicin, Cur: Curcumin.

DOX-treated mice on day 7 (Figure 1D). In light of these results, we conducted an analysis of hearts from GFP-LC3 transgenic mice to investigate the autophagy of the heart. The number of GFP-LC3 dots

was higher in the DOX+Cur group than in the DOX group and was equivalent to the number observed after nutrient starvation for 48 h (Figure 1E). These findings suggest that the cardioprotective effect of Cur when coadministered with DOX is associated with autophagy.

Cur mitigates the proapoptotic effects of DOX in neonatal rat cardiomyocytes

To investigate the effect of Cur on DOX-induced cardiotoxicity, we used neonatal rat cardiomyocytes. Cells were infected with GFP-LC3-expressing adenovirus and incubated for 48 h. An adenovirus expressing the LacZ gene under the same promoter (Ad-LacZ) was used as a control. We treated cells with DOX (1 μ M), Cur (5 μ M), or DOX+Cur (1 μ M and 5 μ M, respectively). After 2 h, the number of GFP-LC3 puncta was higher in DOX+Cur-treated cells than in DOX-treated cells (Figure 2A). Microtubule-associated protein 1 light chain 3-II (LC3-II), a phosphatidylethanolamine-conjugated form of the autophagosomal marker LC3/ β -actin ratio was significantly increased by treatment with DOX+Cur (5 μ M) for 2 h, compared to treatment with DOX alone (Figure 2B) [23]. TUNEL assays showed that apoptotic cell death induced by DOX after 24 h was significantly attenuated by the addition of Cur (DOX vs DOX+Cur, 14.76 ± 0.60 vs. 11.55 ± 1.94,

P<0.05; Figure 2C). Western blot analysis using an antibody specific for PARP showed an increase in cleaved PARP-1 after 24 h of DOX treatment; the increase was attenuated by coadministration of Cur (Figure 2D). These data demonstrate that Cur induces autophagy in myocytes and protects cardiomyocytes from apoptosis induced by DOX.

Inhibition of autophagy negates the cardioprotective effect of Cur

To confirm that the upregulation of autophagy is involved in the antiapoptotic effect of Cur, we administered 3-MA, which blocks autophagosome formation and inhibits autophagy by inhibiting type III phosphatidylinositol 3-kinases [24]. In DOX+Cur-treated cells (1 μ M and 5 μ M, respectively), the addition of 3-MA (10 mM) increased the number of TUNEL-positive cardiomyocytes (DOX+Cur vs DOX+Cur+3-MA, 11.55 \pm 1.12 vs. 17.48 \pm 1.18, *P*<0.05; Figure 3A).



Figure 3: Inhibition of autophagy prevents curcumin from protecting cardiomyocyte. (A) Quantitation of TONEL positive cell nuclei counts of each group. Data shown are averages of eight fields per condition of eight times experiments. Data are mean ± SEM. #p<0.05. (B) Representative western blot analysis to detect cleaved PARP in neonatal rat cardiomyocyte treated with 3MA. Data were normalized with Tubulin which serves as a loading control. Each bar represents mean ± SEM of four independent experiments. (n=4 per group). (C) %FS measured by M-mode echocardiography at 7 days after DOX injection of DOX+Cur-treated mice with chloroquine. Cur was started 5 days prior to DOX. Data are mean ± SEM. (n=6 per group) *p<0.01 #p<0.05. Cont: Control; DOX: Doxorubicin, Cur: Curcumin; NS: Not Significant.

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Cleaved PARP did not decrease in cardiomyocytes treated with DOX (1 μ M) or Cur (5 μ M) with 3-MA, indicating that 3-MA abolished the protective effect of Cur (Figure 3B). To assess the role of autophagy *in vivo*, we intraperitoneally injected mice with chloroquine (20 mg/ kg), which increases the lysosomal pH and disrupts normal autophagic flux. Chloroquine+DOX+Cur-treated mice showed high mortality (86%) in the first week, with significantly decreased systolic cardiac function when compared to DOX+Cur-treated mice (37.4% ± 1.8% vs. 44.4% ± 1.3%, *P*<0.05; Figure 3C, Supplementary Figures 3A and 3B). These data show that the induction of autophagy by Cur reduces DOX-induced cardiotoxicity *in vivo* and *in vitro*.

Cur attenuates DOX-induced cardiotoxicity by regulating autophagy and apoptosis via phosphorylation of JNK

Because Cur induces autophagy and causes cytotoxicity in cancer cells, we hypothesized that the mechanism might be related to the crosstalk between autophagy and apoptosis [11,25]. We examined

Mitogen-Activated Protein Kinase (MAPK) family members, which mediate cellular responses to external stress signals, but could not detect any significant differences in the ERK and p38 pathways in this model (data not shown). Therefore we focused on JNK, a related intracellular signaling molecule. Strong and rapid phosphorylation of INK was observed after DOX treatment. Phosphorylation of INK was suppressed by coadministration of Cur (Figure 4A). Further assessment of cardiomyocytes infected with the GFP-LC3 adenovirus showed that SP600125, a selective JNK inhibitor, attenuated the Curinduced increase in GFP-LC3 positive dots, implying that Cur-induced autophagy was suppressed by JNK inhibition (Figure 4B) [26]. In western blot analysis, pretreatment with SP600125 for 1 h decreased LC3 expression in cells administered DOX and/or Cur (Figure 4C). Cleaved PARP, which was decreased by coadministration of DOX and Cur, relative to treatment with DOX alone, was readily detected in the presence of SP600125 (Figure 4D). These data showed Cur induced autophagy and decreased myocyte apoptosis by reducing the level of DOX induced JNK activation.



Figure 4: Curcumin attenuates doxorubicin cardiotoxicity via regulation of autophagy and apoptosis through JNK phosphorylation. (A) Representative western blot analysis of pJNK, JNK, in post nuclear supernatant of neonatal rat cardiomyocyte. Upper number means loading time of each treatment. Data were normalized with Tubulin which serves as a loading control. Each bar represents mean \pm SEM of four independent experiments. *p<0.01, #p<0.05. (B) Representative confocal photomicrograph of cardiomyocyte transfected with GFP-LC3 treated with starvation, DOX, Cur, DOX+Cur over 2 h respectively. Lower tier cells were treated by SP600125 (JNK inhibitor) 1 h prior to each treatment. (C) Representative western blot analysis of LC3 in neonatal rat cardiomyocyte with JNK inhibitor 1 h prior to each treatment. (C) Representative means \pm SEM of four independent experiments. *p<0.01. (D) Representative western blot analysis to detect cleaved PARP, pJNK, JNK in post nuclear supernatant of neonatal rat cardiomyocyte with JNK inhibitor over 24 h. Tubulin serves as a loading control. Abbreviations are same as in Fig1. Each bar represents mean \pm SEM of four independent experiments. *p<0.01, #p<0.05 NS: Not Significant.

Discussion

Heart failure is the end stage of cardiac disease, a serious pathological condition. Prophylaxis and treatment of heart failure are important aspects of clinical medicine. To investigate approaches for preventing heart failure caused by DOX, we designed a mouse model of DOX-induced cardiotoxicity.

Previous studies have shown that the main mechanism underlying DOX-induced cardiotoxicity is oxidative stress, leading to apoptosis and necrosis, which also transactivates p53 and increases proapoptotic proteins [27-30]. Recently, the cardioprotective effect of curcumin and its antioxidant properties have been reported [31-33]. In this study, we found its remarkable protective effect via induction of cardiac autophagy. Since the first week after the administration of doxorubicin was critical for 28-day mortality, we assumed that effect of curcumin in early days was a key on the survival of mice (Figure 1A). Initial induction of cardiac autophagy by curcumin also reduced the doxorubicininduced heart failure on 28 days. It is also reported that cardiacspecific autophagy-deficient mice develop cardiac failure [34]. Sishi et al. reported that upregulation of autophagy protected against DOXinduced cardiotoxicity [16]. In contrast, DOX-induced cardiomyocyte death was aggravated by the activation of autophagy but attenuated by the inhibition of autophagy through GATA4 gene silencing, suggesting that excessive autophagy contributes to DOX-induced cardiotoxicity [35]. Thus, the role of autophagy in DOX-induced cardiotoxicity is still controversial because autophagy plays roles in both cell protection and cell death. In the present study, we showed that Cur induced autophagy and reduced apoptosis in cardiomyocytes, suggesting that Cur may be a therapeutic target for preventing DOX-induced cardiomyopathy.

Pattingre et al. reported that Bcl-2 functions as an antiapoptotic protein and as an antiautophagy protein through its inhibitory interaction with Beclin-1 [36]. Moreover, marked JNK activity and increased phosphorylation of Bcl-2 were observed in cardiac tissue samples from human heart-failure patients [37]. Recently, novel mechanism of Mst1 mediated Beclin1-Bcl-2 interaction in ischemic heart model has been reported [38]. The molecular mechanisms underlying the interaction between Beclin1 and Bcl-2 by Cur upon DOX induced heart failure model deserve further investigation.

We assessed whether Cur-induced cardiac autophagy was cardioprotective or cardiotoxic. We intraperitoneally administered chloroquine, an autophagy inhibitor, to mice and treated them with DOX+Cur (Supplementary Figure 3). Administration of chloroquine increased mortality, decreased cardiac function, and then abrogated the protective effect of Cur, which indicates that the cardioprotective effect of Cur is mediated by autophagy. We hypothesized that Cur decreased the excessive JNK phosphorylation, and reducing cardiac apoptosis. Strong phosphorylation of JNK resulted in apoptosis, whereas weak phosphorylation of JNK induced autophagy, resulting in cardioprotection (Supplementary Figure 4). When cells were treated with the JNK inhibitor SP600125, the antiapoptotic effect of Cur was abolished (Figure 4D).

According to the previous studies, the peak concentrations of DOX in plasma by standard infusions in patients are 2-20 μ M [39]. In *in vitro* study, we used 1 μ M of DOX in culture model. Although other *in vitro* studies used similar or less dose, this concentration might be lower than the real concentration of the patients' heart tissue [33,40]. We need to be careful in the concentration about the protective mechanism of curcumin in future translational research. Furthermore, since using

actin for a loading control has been recently discussed, the other stable proteins should be checked in future study [23].

In conclusion, our findings indicate that Cur protects cardiomyocytes from DOX-induced apoptosis. JNK plays an important role in the cell fate via the regulation of autophagy by Cur. The mechanism may involve crosstalk between the apoptotic and autophagic machinery mediated by JNK-dependent phosphorylation. These results suggest that Cur has therapeutic potential in inhibiting DOX-induced myocardial damage. Further study is required to determine whether Cur is cardioprotective against other stress to the myocardium.

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