*Full Paper***Doxorubicin Induces Apoptosis by Activation of Caspase-3 in Cultured Cardiomyocytes In Vitro and Rat Cardiac Ventricles In Vivo**

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Abstract. Doxorubicin (DOX) is widely used to treat patients suffering from cancer, but the usage for patients is limited because of the dose-dependent cardiotoxicity. We hypothesized that DOX induces apoptosis through caspase activation in cardiomyocytes, and we examined this hypothesis using both rat primary cultured cardiomyocytes and rat hearts from an animal model. Cardiomyocytes were treated with DOX for 24 h. The activity of caspase-3 was significantly increased by DOX treatment. In rats with DOX injected intravenously once a week for 5 weeks, left ventricular fractional shortening evaluated by echocardiography was significantly decreased at age 14 weeks, 2 weeks after the end of DOX-administration. At 16 weeks of age, endothelin-1 mRNA and atrial natriuretic peptide mRNA were also significantly increased, likewise, and TUNEL positive cells were significantly increased in the ventricles of DOX-treated rats. The activity of caspase-3 in the ventricles was also significantly increased compared to that of untreated rats at 16 weeks. However, the activity of caspase-8 and the expression level of Fas-ligand mRNA were comparable with those of the untreated rats. In conclusion, DOX induces apoptosis through the activation of caspase-3, suggesting that apoptosis has an important role in the progression of cardiomyopathy due to DOX.

Keywords: doxorubicin, apoptosis, cardiomyopathy, caspase, mitochondria

Introduction

The anthracyclin drug doxorubicin (DOX) is one of the most effective antineoplastic agents, and widely used to treat a number of malignancies, including leukemia, lymphoma, and solid tumors. However, its use has been restricted due to the dose-dependent cardiotoxicity, which results in DOX use difficulty, especially in patients at different stages of heart failure (1–3). Several hypotheses were suggested regarding the mechanisms of DOX-induced cardiomyopathy, including impaired myocardial adrenergic regulation (4), intra-

cellular calcium overload (5), and the releases of cardiotoxic cytokines (6). Furthermore, it was reported that free radical oxygen and lipid peroxidation play further important roles in the pathogenesis of DOX-induced cardiomyopathy (7, 8). Recently, it was speculated that DOX-induced the dysfunction of mitochondria, including an impaired calcium handling that may be involved in the myocardial damage (9).

On the other hand, it has been reported that apoptosis plays an important role in the progression of the failing heart in humans (10, 11). It has further been reported that DOX induces apoptosis in cardiomyocytes in specific conditions (12, 13). Moreover, it has been revealed that mitochondrial dysfunction is a critical factor inducing apoptosis (14–17). Therefore, we

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hypothesized that DOX induces apoptosis through caspase activation via the mitochondrial pathway in cardiomyocytes and plays an important role in DOX-induced cardiomyopathy. We examined whether cardiomyocyte apoptosis is induced by DOX using rat primary cardiomyocytes and whether it causes myocardial dysfunction in both cultured cardiomyocytes and ventricles of rat hearts. Furthermore, to clarify the mechanism of DOX-induced cardiomyopathy through apoptosis, we investigated whether the activity of the caspase enzymes, a protease responsible for cell death, is affected by DOX.

Materials and Methods

Study protocols – in vitro

Primary cultured cardiomyocytes were prepared from the hearts of neonatal Sprague-Dawley rats, as previously reported (18). After 24-h serum starvation, 10^{-6} M doxorubicin (DOX) was added to culture medium. The dose of DOX was determined from our preliminary study. Apoptosis was assessed by the detection of DNA laddering, and the activity of caspase-3 and caspase-8 was measured by fluorometric protein assay methods.

Study protocols – in vivo

Eight-week-old male Sprague-Dawley rats were used. Treatment with DOX (Kyowa Hakko Kogyo Co., Ltd., Tokyo) was performed as previously reported; 3 mg/kg DOX ($n = 15$) or saline ($n = 15$) was injected through the tail vein weekly for 5 weeks (19). In the preliminary study in that report, this protocol resulted in the most severe heart failure model, because the protocol of 3 times injection of DOX at 5 mg/kg per week resulted in severe cardiac damage, but 7 times with DOX at 2 mg/kg per week showed only slight cardiac damage (19). Five rats were evaluated respectively at week 0 (12-week-old), 2 weeks (14-week-old), and 4 weeks (16-week-old) after the treatment of DOX. After evaluating the cardiac function with echocardiography, the heart and lungs were excised. The ventricles were divided into several pieces; one of the pieces was immediately measured for caspase activity. To evaluate the apoptosis by TdT-mediated dUTP-biotin nick and labeling (TUNEL) method, another piece was immersed in 10% buffered formalin. The other pieces were rapidly frozen in liquid nitrogen, and stored at -80°C .

Primary culture of rats cardiomyocytes

Primary culture of rat neonatal cardiomyocytes was performed as demonstrated in our previous studies (18, 20). Hearts were excised from 2 to 3-day-old neonatal

Sprague-Dawley rats. The ventricles were minced in Hepes buffer (30 mM Hepes, 120 mM NaCl, 4 mM dextrose, 2 mM KCl, 1 mM KH_2PO_4 , pH 7.6). The ventricles were incubated in 0.2% collagenase (Wako Pure Chemical, Tokyo) for 45 min at 37°C . Cardiac cells were collected and suspended in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Bio Whittaker, Walkersville, MD, USA), 100 U/ml penicillin, and $100\text{ }\mu\text{g/ml}$ streptomycin (Meiji Seika, Tokyo). After differential adhesion, the floating cardiomyocytes were collected and then incubated in DMEM/F-12 containing ITS-X supplement (Gibco BRL) on fibronectin-coated dishes. More than 90% of cells were identified as cardiomyocytes by the detection of cross-striation structures in the cells stained with Bodipy-Phalloidin (Molecular Probes, Eugene, OR, USA). Cells were cultured for 1 day after differential adhesion and then used for further experiments.

DNA laddering

Cardiomyocytes were scraped and collected by centrifugation. The cell pellets were lysed by $100\text{ }\mu\text{l}$ lysis buffer (10 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100) for 10 min on ice, and centrifuged. The supernatant of the cell lysate was incubated with RNase A for 30 min at 37°C , followed by incubation with proteinase K for 1 h at 37°C . Then DNA was extracted and resuspended in TE buffer. The DNA samples were subjected to 1.2% agarose gel electrophoresis and stained with ethidium bromide for visualization under UV light.

Two-dimensional echocardiography

Two-dimensional echocardiography of rats was performed with modifications of the previously reported methods (21). On the day of the evaluation, the rat was anesthetized with sodium pentobarbital (50 mg/kg). The chest was shaved, and two-dimensional echocardiography was performed with the echocardiographic systems (model SSD-900; Aloka, Tokyo) and 7.5 MHz probe (UST-987-7.5, Aloka). M-mode echocardiograms of the left ventricle (LV) at the papillary muscle level were determined, guided by two-dimensional short-axis images. End-diastolic posterior wall thickness, end-diastolic and end-systolic internal diameters of the LV were measured by a single observer. Relative wall thickness (RWT) was calculated as follows:

$$\text{RWT} = 2 \times \text{LVPWTd} / \text{LVDD},$$

where LVPWTd is an end-diastolic posterior wall thickness of LV, and LVDD is an end-diastolic internal diameter of LV. Fractional shortening (FS) was calcu-

lated as follows:

$FS = 100 \times (LVDd - LVDs) / LVDd$,
where LVDs is an end-systolic internal diameter of the LV.

Measurement of caspase-3 and -8 activities

Several fold increases in caspase-3 and caspase-8 activities were determined using an assay kit (Medical & Biological Laboratory, Nagoya) (22). Caspase-3 activity was measured by detection of the cleavage of the substrate 7-amino-4-trifluoromethyl coumarin conjugated to Asp-Glu-Val-Asp (DEVD-AFC). In the case of caspase-8 activity, 7-amino-4-trifluoromethyl coumarin conjugated Isoleucin-Glu-Thr-Asp (IETD-AFC) was used for the substrate. Briefly, 5×10^5 cardiomyocyte cells were scraped and lysed, and then the lysates were incubated with the substrate for 1 h at 37°C. Subsequently, the intensity of fluorescence was measured by a spectrophotometer, Bio Lumin 960 (Molecular Dynamics, Sunnyvale, CA, USA), with an excitation wave length of 405 nm and an emission wave length of 505 nm. The activity was determined as an increase by comparison with the level of the untreated control. In the case of the in vivo study, the tissue was homogenized in cell lysis buffer with a tissue homogenizer. Homogenized tissue was washed with lysis buffer, and 250 μ g of these cell lysates were used for the assay. The lysates were incubated in the same manner as the in vitro study. The caspase activity was determined as an increase in comparison with the level of the untreated control.

RT-PCR to evaluate the expression of atrial natriuretic peptide (ANP) mRNA, endothelin-1 (ET-1) mRNA, Fas ligand (FasL) mRNA, and β -actin mRNA in the heart

The expression levels of ANP mRNA, ET-1 mRNA, and FasL mRNA were analyzed by RT-PCR. The evaluation using a RT-PCR method was performed according to our previous report (23). The expression of β -actin mRNA was also determined as an internal control.

Total RNA was isolated by ISOGEN (Nippon Gene Ltd., Tokyo), treated with DNase I (TaKaRa Ltd., Otsu), and extracted again using ISOGEN to eliminate the genomic DNA. Total RNA (5 μ g) was primed with 0.05 μ g oligo-d(T)₁₂₋₁₈ and reverse transcribed using Avian Myeloblastosis virus reverse transcriptase (Life Sciences, Inc., FL, USA). The cDNA was diluted in a 1:10 ratio, and 1 μ l was used for PCR. The gene-specific primers were synthesized according to the published cDNA sequences for each of the following: ANP, ET-1, FasL, and β -actin. The sequences of the oligonucleotides were as follows:

ANP (sense): 5'ATGGGCTCCTTCTCCATCACC3',

ANP (antisense):

5'TCCGCTCTGGGCTCCAATCCTGT3',

preproET-1 (sense):

5'TCTTCTCTCTGCTGTTTGTG3',

preproET-1 (antisense):

5'TTAGTTTTCTTCCCTCCACC3',

FasL (sense): 5'CAGCCCCTGAATTACCCATGTC3',

FasL (antisense):

5'CACTCCAGAGATCAAAGCAGTTC3',

β -actin (sense): 5'GAAGATCCTGACCGAGCGTG3',

β -actin (antisense):

5'CGTACTCCTGCTTGCTGATCC3'.

The annealing temperatures were set as follows: ANP, 65°C; ET-1, 54°C; FasL, 66°C; and β -actin, 72°C. The reaction cycles of PCR were performed in the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products. The PCR products were found to be of the expected size as shown by 1.2% agarose gel electrophoresis. In addition, the specificity of the amplified sequences was confirmed by restriction enzyme analysis and DNA sequencing.

Semiquantitative analysis of PCR products

The amplified PCR products were visualized by a UV transilluminator and photographed. The ratios of ANP mRNA, preproET-1 mRNA, FasL mRNA to β -actin mRNA were calculated. Quantification was performed by a personal computer with MacBAS software (Fuji Film Ltd., Tokyo) according to our previous paper (20). Thus, the level of expression of each gene was normalized by that of β -actin.

TUNEL staining

TUNEL staining was performed according to the manufacturer's instructions (24). The tissue was fixed in 10% buffered formalin and embedded in paraffin. The section was treated with 250 μ l of proteinase K (PK) solution, followed by a treatment with 50 μ l terminal deoxynucleotidyl transferase (TdT) buffer. After removing TdT buffer, the section was reacted with 50 μ l TdT solution (mixture of 45 μ l of TdT buffer, 2.5 μ l of FITC-dUTP, and 2.5 μ l of TdT). After removing TdT solution, the slide was mounted with mounting medium (90% glycerol, 10% PBS) and observed by fluoroscopy.

Statistical analyses

All data were presented as mean $\pm$ S.E.M. values. All statistical comparisons were performed with a commercially available statistical package for Macintosh personal computer (STAT VIEW, version 4.5; Abacus Concepts, Inc., Berkeley, CA, USA). The significant differences between two values was analyzed by Kruskal-Wallis one-way analysis of variance

(ANOVA), followed by Fischer's PLSD for multiple comparisons. The results were considered statistically significant at the level of $P < 0.05$.

Results

DOX induces apoptosis in cultured cardiomyocytes through activation of caspase-3 (in vitro study)

Rat primary cardiomyocytes were treated with 10^{-6} M DOX. DNA laddering was initially revealed to be evident after 12-h DOX treatment (Fig. 1). With 24-h DOX treatments, DNA laddering was markedly increased, compared with that without DOX (control) (Fig. 1). The activity of caspase-3 or caspase-8 was evaluated as a relative increase in comparison with the level of the non-treated cells. Caspase-3 activity had

increased by 6 h of DOX-treatment, consequently preceding initial DNA laddering. Furthermore, caspase-3 activity was increased in a time-dependent manner to up to 6 times of the control level after the 21 h of DOX-treatment (Fig. 2). In comparison, caspase-8 activity was not remarkably affected by DOX (Fig. 2).

DOX decreases cardiac function with increased expression of both ANP mRNA and ET-1 mRNA (in vivo study)

FS of LV, as an indicator of pump function evaluated by echocardiography, is shown in Fig. 3A. In rats with DOX, FS was significantly decreased at age 14 weeks (2 weeks after the end of DOX-administration) and FS was markedly diminished at age 16 weeks (Fig. 3A). The changes in RWT of the LV, as assessed by echocardiography, are shown in Fig. 3B. In rats with DOX,

Fig. 1. Electrophoretic pattern of DNA extracted from cardiomyocytes with or without doxorubicin (DOX). DNA laddering was detected 12 h after the exposure to DOX. With 24-h treatment of DOX, DNA laddering was further increased, compared with DOX untreated cardiomyocytes. CTL: cardiomyocytes without DOX, DOX: cardiomyocytes with DOX (10^{-6} M).

Fig. 2. The activities of caspases-3 and -8 in cardiomyocytes with or without doxorubicin (DOX). The activities of caspase-3 and caspase-8 was determined to increase, in comparison with the level of the untreated control cells. White bars: Cardiomyocytes without DOX, Solid bars: Cardiomyocytes with DOX (10^{-6} M). Each column and bar represent the mean $\pm$ S.E.M. * $P < 0.05$ vs cardiomyocytes without DOX.

Fig. 3. The left ventricular fractional shortening (A) and the relative wall thickness (B) of rats with or without doxorubicin (DOX). White bars: rats with saline ($n = 5$). Solid bars: rats with DOX treatment ($n = 5$). Each column and bar represented the mean $\pm$ S.E.M. * $P < 0.05$ vs rats with saline.

Fig. 4. The ratio of the ventricular weight to body weight and the ratio of the lung weight to body weight of rats with or without doxorubicin (DOX) treatment. A: The ventricular weight to body weight. B: The lung weight to body weight. White bars: rats with saline ($n = 5$). Solid bars: rats with DOX treatment ($n = 5$). Each column and bar represented the mean $\pm$ S.E.M. * $P < 0.05$ vs rats with saline.

RWT was significantly decreased at age 14 weeks and was still further reduced at age 16 weeks (Fig. 3B). Comparable with impairment of the heart, there was a gain in the ratio of the ventricular weight to body weight in rats treated with DOX, whereas this was not observed in untreated control rats (Fig. 4A), and additionally, the ratio of lung weight to body weight was significantly increased in DOX treated rats more than control rats (Fig. 4B).

Fig. 5. The gene expression levels of atrial natriuretic peptide (ANP) mRNA (A) and ET-1 mRNA (B) in the ventricle of rats with or without doxorubicin (DOX) treatment. The expression of β -actin mRNA was shown as an internal control. The ratios of ANP mRNA (A) and preproET-1 mRNA (B) to β -actin mRNA were calculated. White bars: rats with saline ($n=5$). Solid bars: rats with DOX treatment ($n=5$). Each column and bar represented the mean $\pm$ S.E.M. * $P < 0.05$ vs rats with saline.

Fig. 6. TUNEL staining in doxorubicin (DOX)-treated ventricles. A: Typical photomicrographs of in situ TUNEL staining in ventricles of 16-week-old rats with or without DOX treatment. Arrow indicates labeled myocardial nuclei. Magnification $\times 400$. B: Bar graphs showing numbers of TUNEL positive cells in ventricles of 16-week-old rats with or without DOX treatment. White bars: rats with saline ($n=5$). Solid bars: rats with DOX treatment ($n=5$). Each column and bar represent the mean $\pm$ S.E.M. * $P < 0.05$ vs rats with saline.

ANP mRNA and ET-1 mRNA were evaluated by RT-PCR. The expression of ANP mRNA in the ventricles of rats treated with DOX was significantly increased at age 12 weeks, followed by a gradual increase during the time course (Fig. 5A). On the other hand, the expression level of ET-1 mRNA increased only in the initial period between the ages 14 to 16 weeks (Fig. 5B).

DOX induces apoptosis in the whole heart via activation of caspase-3

To evaluate the apoptosis of myocardium in the ventricles during the progression of DOX-induced heart failure, we performed TUNEL staining. TUNEL

Fig. 7. The activity of caspases-3 and -8 from rat ventricles treated with or without doxorubicin (DOX). The activity of caspase-3 and caspase-8 was determined relative to the level of the control. White bars: rats with saline ($n=5$). Solid bars: rats with DOX treatment ($n=5$). Each column and bar represent the mean $\pm$ S.E.M. * $P < 0.05$ vs rats with saline.

Fig. 8. Comparison of gene expression level of Fas-ligand mRNA between the ventricle of rats with or without doxorubicin (DOX) treatment. The expression of β -actin mRNA is shown as an internal control. The ratios of Fas-ligand mRNA to β -actin mRNA were calculated. White bars: rats with saline ($n=5$). Solid bars: rats with DOX treatment ($n=5$). Each column and bar represent the mean $\pm$ S.E.M. The data were not significant between rats with saline and rats with DOX at each time.

positive ventricle cells were significantly increased (2.5 times more than that in untreated rats) in DOX-treated rats at the age of 16 weeks (Fig. 6: A and B). At the age of 16 weeks, the activity of caspase-3 in the ventricles of DOX-treated rats was significantly increased over untreated rats, but the activity of caspase-8 was not markedly increased (Fig. 7). Likewise, the expression of FasL mRNA in DOX-treated rats was not increased, but was comparable with that in untreated control rats (Fig. 8).

Discussion

The present study revealed the following: i) in the cardiomyocytes, apoptotic DNA laddering was induced by DOX; and associated with that, the activity of caspase-3 was significantly increased, but the activity of caspase-8 was not markedly increased. ii) In DOX treated rats, left ventricular function had markedly deteriorated with increased expression of a cardiac neurohormonal genes. In those rats, FS of LV was markedly decreased and the RWT of the LV was significantly reduced. It was suggested that the reduction of RWT with DOX is due to an increase in wall stress to LV, and it is associated with an impaired cardiac function. The ratio of lung weight to body weight was significantly increased in DOX-treated rats, suggesting pulmonary congestion due to heart failure. The expressions of ANP mRNA and ET-1 mRNA in the ventricles of rats treated with DOX were significantly increased. It is suggested that DOX-treated hearts demonstrate a heart failure-specific gene expression pattern during aggravation of cardiac function. Apoptosis in the hearts of DOX-treated rats was detected by TUNEL staining. The activity of caspase-3 in the ventricles of DOX-treated rats was increased; however, the expression of FasL mRNA and the activity of caspase-8 were unchanged. These results suggested that DOX induces apoptosis in cardiomyocytes through the activation of caspase-3, without any significant activation of caspase-8.

In previous studies, it was reported that apoptosis was detected during heart failure in experimental animal models (13, 25) and patients (10, 11). Narula et al. reported that apoptosis could be detected using TUNEL methods in myocardial specimens from patients with dilated cardiomyopathy, who were undergoing cardiac transplantation (10). From these reports, it is understood that apoptosis has an important role in the progression of heart failure, although apoptosis is not the only one pathogenesis of heart failure and it is still not clear whether apoptosis is the main cause of the cellular loss in the failing heart. As demonstrated in the present study,

DOX induces apoptosis in rat cardiomyocytes, and it is thus suggested that apoptosis plays a crucial role in the progression of myocardial dysfunction induced by DOX. Concomitantly, cardiac ANP mRNA and ET-1 mRNA were respectively increased during this period of initial cardiac dysfunction. Consequently, with a reduction in FS, DOX further depressed cardiac function, and this cardiac malfunction manifested itself with an increase in gene expression of ET-1 mRNA, which is another marker of heart failure.

As highlighted in the present study, DOX impaired the cardiac function via activation of caspase-3 without caspase-8. It is known that there are several possible pathways involved in apoptosis. One of the important pathways is the death receptor pathway mediated by Fas/FasL. TNF- α and FasL are known to activate this pathway through caspase-8. Another pathway is mediated by mitochondria. Impairment of mitochondria leading to collapse of mitochondrial membrane potential causes release of cytochrome C and activates caspase-9 leading to activation of caspase-3. Then intranucleosomal DNA fragmentation is finally induced by endonucleases and the final steps of apoptosis occur (14, 26). In the previous study, Childs et al. reported that DOX finally induces oxidative stress. It leads to opening of mitochondrial membrane permeability transition pore and the release of proapoptotic proteins including cytochrome C from the mitochondrial matrix. The release is known to be caused by mitochondrial dysfunction because the membrane potential could not be properly regulated in such a case. Cytochrome C activates caspase-3 through the interaction with Apaf-1 and caspase-9, resulting in apoptosis (27). In the present study, caspase-3 was at least activated. Therefore, it is suggested that mitochondrial function might be impaired. Therefore, it is conceivable that DOX-induced apoptosis is mediated through caspase-3 activation through the mitochondrial pathway.

In the past, we reported cytochrome C released from mitochondria has an important role in apoptosis and cardiac dysfunction in cultured cells (18, 20). Furthermore, it was reported that apoptosis is an important mechanism for the loss of myocardial cells during cardiac dysfunction (11). In another study using a human specimen, the release of cytochrome C from mitochondria and activation of caspase-3 were observed in the heart from cardiomyopathy (28). In previous reports, as mentioned above, it was suggested that DOX induces oxidative stress and mitochondrial-mediated apoptosis via cytochrome C release (27, 29). Moreover, it was reported that DOX-induced cardiotoxicity is related to the generation of highly reactive oxygen species and the poor antioxidant defense system of the heart (30).

However, so far, the mechanism of induction of apoptosis in DOX-induced cardiomyopathy has not been well clarified. Therefore, our present study presents meaningful insight into the pathophysiology of DOX-induced cardiomyopathy. From the results of this present study, it is considered that DOX is intimately involved in caspase activation via a mitochondrial pathway. However, there are some reports about mechanisms of DOX-induced apoptosis suggesting that apoptosis is induced by Fas and FasL in rat cardiomyocytes (31, 32) and the mitogen-activated protein kinase family (33). DOX induces an accumulation of ceramide causing apoptosis (34), or non-cardiomyocytes inhibit DOX-induced cardiomyocyte-apoptosis via ET-1 activation (35). In the present study, DOX did not affect the expression of FasL mRNA. The reason for the discrepancy from the previous reports (31, 32) has not yet been determined, but it might be due to the different severity of the heart injury produced by DOX in a different dose and protocol using DOX. Therefore, many pathways might be speculated to be responsible for apoptosis induction and there may be cross talk between these various pathways, including the mitochondrial pathway through caspase-3 activation.

In conclusion, it is suggested that apoptosis induced by caspase-3 activation has an important role in the progression of cardiomyopathy due to DOX. Further investigations to clarify the mechanism of apoptosis in cardiomyopathy due to DOX might be expected to prevent cardiac injury.

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