The mRNA expression of *Il6* and *Pdcd1* are predictive and protective factors for doxorubicin-induced cardiotoxicity

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Abstract. Anthracyclines, such as doxorubicin (DOX), have been widely used in the treatment of a number of different solid and hematological malignancies. However, these drugs can inflict cumulative dose-dependent and irreversible damage to the heart, and can occasionally lead to heart failure. The cardiotoxic susceptibility varies among patients treated with anthracycline, and delays in the recognition of cardiotoxicity can result in poor prognoses. Accordingly, if the risk of cardiotoxicity could be predicted prior to drug administration, it would aid in safer and more effective chemotherapy treatment. The present study was carried out to identify genes that can predict DOX-induced cardiotoxicity (DICT). In an *in vivo* study, mice cumulatively treated with DOX demonstrated increases in serum levels of cardiac enzymes (aspartate aminotransferase, lactate dehydrogenase, creatine kinase MB isoenzyme and troponin T), in addition to decreases in body and heart weights. These changes were indicative of DICT, but the severity of these effects varied among individual mice. In the current study, the correlation in these mice between the extent of DICT and circulating blood concentrations of relevant transcripts before DOX administration was analyzed. Among various candidate genes, the plasma mRNA levels of the genes encoding interleukin 6 (*Il6*) and programmed cell death 1 (*Pdcd1*) in blood exhibited significant and positive correlations with the severity of DICT. In an *in vitro* study using cardiomyocyte H9c2 cells, knockdown of *Il6* or *Pdcd1* by small interfering RNA was revealed to enhance DOX-induced apoptosis, as determined by luminescent assays. These results suggested that the levels of transcription of *Il6* and *Pdcd1* in cardiomyocytes serve a protective role against DICT, and that the accumulation of these gene transcripts in blood is a predictive marker for DICT. To the best of our knowledge, this is the first report to demonstrate a role for *Il6* and *Pdcd1* mRNA expression in DICT.

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Abbreviations: DOX, doxorubicin; ACE, angiotensin-converting enzyme; ARBs, angiotensin II receptor blockers; PDCD1, programmed cell death 1; Sod3, superoxide dismutase-3; Gpx3, glutathione peroxidase-3; Mt1, metallothionein-1; DICT, DOX-induced cardiotoxicity; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; CK-MB, creatine kinase MB isoenzyme

Key words: doxorubicin, interleukin 6, programmed cell death 1, cardiotoxicity, gene expression, apoptosis

Introduction

Anthracyclines are chemotherapy drugs that inhibit the activity of topoisomerase IIα, thereby negatively impacting DNA synthesis in cancer cells; anthracyclines have been widely used in the treatment of various solid and hematological malignancies (1,2). It is well known, however, that anthracyclines can inflict severe damage to the heart, and cardiotoxicity represents one of the most important adverse effects of this class of compounds (1-4). Anthracycline-induced cardiotoxicity is cumulative, dose dependent, and irreversible; therefore, these drugs increase the risk of heart failure, in turn leading to increased mortality and decreased quality of life in patients (1-4). Although the precise mechanism of cardiotoxicity is not fully understood, cardiac apoptosis (programmed cell death) mediated by either of the following two mechanisms has been proposed: One is the excessive production of reactive oxygen species, the other is DNA damage by inhibition of topoisomerase IIβ in cardiomyocytes (1,2,5).

Retrospective pooled analysis by Swain *et al* (3) documented that rates of heart failure caused by doxorubicin (DOX), a typical anthracycline drug, were 5, 26 and 48% at cumulative doses of 400, 500 and 700 mg/m^2, respectively, providing a rationale for limiting the cumulative lifetime dose of DOX. Nevertheless, there are individual differences in the maximal cumulative dose tolerated, and anthracyclines can lead to the occurrence and development of heart failure even at lower doses (5-7). In addition, the risk of heart failure may be augmented by pre-existing loading conditions such as hypertension and valvular disease (8), and by combination with other chemotherapy drugs such as molecularly targeted drugs and immune checkpoint inhibitors (9). These facts indicate that anthracyclines have no safe treatment dose.

Several strategies for reducing the risk of heart failure in anthracycline-treated patients have been proposed, including

pharmacological therapy to prevent cardiotoxicity (10). Although some antioxidants, such as vitamin E and probucol, have been observed to provide cardioprotective action in DOX-treated animal models (2,11), there is (to date) no direct evidence supporting clinically efficacy (12). Alternatively, it has been demonstrated (in both animals and humans) that standard drugs for heart failure, such as β-blockers and angiotensin-converting enzyme (ACE) inhibitors/angiotensin II receptor blockers (ARBs), are effective in attenuating DOX-induced cardiac damage (2,10,13). In fact, pharmacological therapy with these drugs is generally accepted as a preventive strategy against cardiac dysfunction in anthracycline-treated patients (10). It should be noted, however, that the response rate to the pharmacological therapy progressively decreases with increasing time delay between the end of chemotherapy to the onset of cardiac dysfunction (i.e., with delayed start of the pharmacological therapy) (13,14). The delayed recognition of cardiotoxicity results in a poor prognosis, presumably because of the accumulation of irreversible cardiac dysfunction and a lack of clinical response to the pharmacological therapy. Therefore, early detection of cardiac abnormalities and early adequate therapy would be extremely important in preventing the occurrence and development of heart failure after anthracycline therapy (15).

If we could predict the risk of cardiotoxicity prior to the administration of anthracycline, it would help to render chemotherapy in cancer patients safer and more effective. Genetic factors have been shown to determine individual susceptibility to DOX-induced cardiotoxicity (DICT) (16,17). Therefore, in the present study, we identified genes closely associated with DICT (i.e., predictive biomarkers) in mice *in vivo*, and then examined the roles of these genes in DOX-induced apoptosis of H9c2 cells (rat cardiac myoblasts) *in vitro*.

Materials and methods

Animals and chemicals. Four-week-old adult male C57BL/6J mice (22-24 g each), were obtained from Japan SLC (Hamamatsu, Japan). The animals were maintained on a 12-h/12-h light/dark cycle in a temperature- and humidity-controlled room. Experiments were conducted in accordance with the standards established by the Japanese Pharmacological Society and were approved by the Tohoku Medical and Pharmaceutical University of Institutional Animal Care and Use Committee (Experimental Protocol no. 18013). Animals were allowed free access to laboratory pellet chow (CE-2; CLEA Japan, Inc.) and water throughout the experiments. DOX was purchased from Sandoz K. K. All other reagents, unless stated, were of the highest grade available and were supplied by either Sigma Chemical Co. or Wako Pure Chemical Industries, Ltd.

DOX administration and collection of samples. One week before the start of dosing, 50 μl of blood was collected from a cut on the tail vein of each mouse, and total RNA was extracted for reverse transcription-quantitative PCR (RT-qPCR). Mice were injected intraperitoneally with 3.33 mg/kg DOX every other day for 18 days, resulting in a cumulative DOX dose of 30 mg/kg. This cumulative dose of DOX in mice is estimated to be comparable to approximately 100 mg/m^2 in humans (18), which is considerably lower when compared to clinical cardiotoxic doses (3). However, the cumulative dose of DOX we used is sufficient to cause damage to the murine heart, as supported by many other studies (19-21); thus, the susceptibility to DICT may be greater in mice than in humans. The control mice were injected intraperitoneally with an equivalent volume of saline. Animals were euthanized by exsanguination via jugular puncture on the seventh day after the last DOX administration under inhalation anesthesia with 2% isoflurane; whole blood and heart samples were collected. A small portion of whole blood was transferred into ethylenediaminetetraacetic acid (EDTA)-coated tubes and used for measurement of hematological parameters. The remaining blood was immediately centrifuged at 1,000 x g for 10 min at 4˚C to separate serum for measurement of cardiac injury parameters. Heart samples were immediately frozen and stored at -80˚C until used for RNA isolation. As an index of cardiac hypertrophy, the body weight-normalized heart weight was calculated.

Measurements of hematological parameters and serum cardiac injury parameters. As hematological parameters, white blood cells (WBCs), red blood cells (RBCs) and platelets were counted, and hemoglobin (Hb) levels and hematocrit (Hct) were assessed using a hematology analyzer (EYM-230; Erma, Inc.). As indices of cardiac injury, serum levels of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), creatine kinase MB isoenzyme (CK-MB), and troponin T were determined; the first two were measured using a colorimetric kit (Wako) as described in our previous reports (22,23), and the last two were measured using a diagnostic kit (Cloud-Clone Corp.) according to the supplier's instructions.

Cell culture. Rat heart-derived embryonic myoblast H9c2 (2-1) cells were obtained from DS Pharma Biomedical. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2-95% air incubator under standard conditions. Staining with 0.2% Trypan blue was performed to determine viable cell counts. To maintain exponential growth, cells were seeded at a density of 5x10^4 cells/ml and were passaged every 3-4 days. For the remaining assays cells were cultured in 2-ml aliquots in 35-mm dishes.

RNA extraction and RT-qPCR. Total RNA was isolated from blood using a NucleoSpin® RNA blood kit (MACHEREY-NAGEL GmbH & Co. KG) according to the manufacturer's instructions. Total RNA was purified from mouse heart and H9c2 cells by extraction using ISOGEN reagent (Nippon Gene) according to the manufacturer's protocol. The quantity and the purity of extracted RNA were determined on a NanoDrop ND-1000 spectrophotometer. RNA samples with 260/280 ratios higher than 1.8 were used for experiments. Total RNA (100 ng) was converted into cDNA using the ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan). Aliquots of the resulting cDNA preparations then were subjected to qPCR analysis using the KOD SYBR® qPCR Mix (Toyobo). A CFX Connect™ Real-Time PCR system (Bio-Rad Laboratories, Inc.) was used to determine mRNA expression levels of the genes encoding connective tissue growth factor

Table I. Primers used for reverse transcription-quantitative PCR (RT-qPCR) analysis.

A, Mouse

Gene	GenBank accession number	F primer sequence (5'-3')	R primer sequence (5'-3')
Gpx3	NM_008161.3	TCAACGTAGCCAGCTACTGAGGTC	CTGTTTGCCAAATTGGTTGGAA-
Il6	NM_031168.1	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
Mt1	NM_013602.3	TCTAAGCGTCACCACGACTTCA	GTGCACTTGCAGTTCTTGCAG
Pdcd1	NM_008798.2	TGGCAATCAGGGTGGCTTC	GACTCAGGCGGTTCCAGTTCA
Sod3	NM_011435.3	GTGTCCCAAGACAATCCCACAA	GGGAGTACTCTCAAAGGTGCTCA
Ctgf	NM_010217.2	ACCCGAGTTACCAATGACAATACC	CCGCAGAACTTAGCCCTGTATG
Gapdh	NM_008084.3	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

B, Rat

Gene	GenBank accession number	F primer sequence (5'-3')	R primer sequence (5'-3')
Il6	NM_012589.2	ATTGTATGAACAGCGATGATGCAC	CCAGGTAGAAACGGAACTCCAGA
Pdcd1	NM_001106927.1	GCGCTTGCACTGTTGAGTGAG	TGCCCAACAATAGGATTCAGGAG
Gapdh	NM_017008.4	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

F, forward; R, reverse; *Gpx3*, glutathione peroxidase-3; *Mt1*, metallothionein-1; *Pdcd1*, programmed cell death 1; *Sod3*, superoxide dismutase-3; *Ctgf*, connective tissue growth factor.

(*Ctgf*), interleukin 6 (*Il6*), programmed cell death 1 gene (*Pdcd1*, also called PD-1), superoxide dismutase-3 (*Sod3*), glutathione peroxidase-3 (*Gpx3*), and metallothionein-1 (*Mt1*). The primer pairs used were obtained from the Takara Perfect Real Time Primers collection (Takara Bio). cDNA/Taq-polymerase was denatured at 98˚C for 2 min, and then 40 amplification cycles (each cycle: At 98˚C for 10 sec, at 60˚C for 10 sec, and at 68˚C for 30 sec) were performed. The primer sequences and GenBank accession numbers are listed in Table I. Transcript levels were normalized to those of the housekeeping gene *Gapdh* (encoding glyceraldehyde-3-phosphate dehydrogenase) using the following formulae: ΔCq=Cq target-Cq reference; and ΔΔCq=mean value of ΔCq sample-ΔCq control. Finally, the $2^{-\Delta\Delta Cq}$ method (24) was used to calculate the differences in mRNA transcription levels. The results of all assays were checked against melting curves to confirm the presence of single PCR products. At least two independent experiments were conducted and samples were assessed in (at least) triplicate in each experiment.

Il6 and Pdcd1 knockdown. Small interfering RNA (siRNA)-*Il6* (siIl6) and siRNA-*Pdcd1* (siPdcd1) were transfected into H9c2 cells using the HyperFect transfection reagent (Qiagen) according to the manufacturer's protocol. A non-targeting siRNA (Mock) was used as a vehicle control to assess the non-sequence-specific effects of transfected siRNAs. The siRNAs used were siIl6, a FlexiTube siRNA (ID no. SI01525356, Qiagen) and siPdcd1, a Silencer® Select Pre-designed siRNA Product (ID no. s154115, Thermo Fisher Scientific, Inc.); the negative control siRNA was obtained as AllStars Neg. Control siRNA (ID no. AM4611, Qiagen). Transfections consisted of $4x10^4$ cells combined with a given siRNA (final concentration, 10 nM) in the presence of HyperFect reagent; the mixtures then were incubated for 24 h before assessment of *Il6* or *Pdcd1* expression.

IL-6 and PDCD1 immunofluorescence. Cells transfected with siRNAs were seeded into the Lab-Tek® 8-well chambered cover glass system plates (Thermo Fisher Scientific, Inc.) at $4x10^4$ cells/ml and incubated overnight under standard culture conditions. The chambered slides were washed twice with phosphate-buffered saline (PBS) adjusted to pH 7.4 and fixed in ice-cold 1:1 methanol:acetone for 30 min. The slides were immersed for 10 min in 1% goat serum and 0.25% Triton X-100 in PBS and then transferred to Blocking One Histo (Nacalai Tesque) for 10 min. The slides then were washed with PBS containing 0.1% Tween-20, incubated with primary antibody anti-IL-6 rabbit polyclonal antibody (cat. no. NB600-1131, Novus Biologicals) or anti-PDCD1 mouse monoclonal antibody (cat. no. 66220-1-Ig, Proteintech®) at 1:1,000 in PBS for 1 h at room temperature, washed with PBS, and incubated with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific, Inc.) for 1 h. After rinsing with PBS, a drop of UltraCruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, Inc.) was added to each well. Cells were observed under a confocal fluorescence microscope C-1

(Nikon) and cell components were visualized by fluorescent intensity in blue (405 nm) for nuclear DNA, green (488 nm) for PDCD1-positive cells, and red (594 nm) for IL-6-positive cells.

Apoptosis assay. Annexin V-mediated detection of phosphatidylserine (PS) was used to identify cells at early stages of apoptosis, because the redistribution of PS from the internal membranes to the external membrane surface occurs in early apoptosis (25). Several previous reports have employed an annexin V assay to determine apoptosis in DOX-treated cardiomyocytes (26,27). On the other hand, caspase-3/7 is known to be a key downstream effector in the apoptosis pathway (28). To test both aspects of cell death, we assessed the DOX-induced apoptosis using the RealTime-Glo™ Annexin V Apoptosis assay (Promega Corp.) and the Caspase-Glo® 3/7 Assay (Promega Corp.). Briefly, the H9c2 cells were transfected with siRNAs, distributed in a 96-well plate at a density of $4x10^3$ cells per well, and allowed to adhere overnight. For the RealTime-Glo™ Annexin V Apoptosis assay, the adhered cells were incubated with 1 μM DOX in the presences of annexin V-luciferase reagents and time-dependent increases in luminescence were monitored, reflecting the apoptotic process. Similarly, apoptosis was evaluated after incubation for 24 h with the concentration of DOX at 0.01, 0.03, 0.1, 0.3 or 1 μM. These concentrations were set based on the results of a previous study (29), in which DOX at 0.1 μM or higher was shown to induce apoptosis of cardiomyocyte cells. For the Caspase-Glo® 3/7 Assay, the adhered cells were incubated with DOX at 0.1, 0.3 or 1 μM for 4 or 8 h; Caspase-Glo® 3/7 reagents then were added to each well and the contents were gently mixed. The resulting luminescent intensity was measured using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). Differences between two groups were compared using one-way analysis of variance (ANOVA). Multiple comparisons were performed by one-way ANOVA followed by Tukey post hoc test. P-value of less than 0.05 was considered significant. Correlation analyses were performed using the Spearman's rank correlation coefficient method. Statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Ltd.).

Results

DICT and DOX-induced hematotoxicity. Toxic effects of DOX on the heart and in blood cells were evaluated in mice treated with a cumulative dose of 30 mg/kg. The body weight and heart weight of DOX-treated mice were almost 30% lower than those of the control mice (Fig. 1A and B), whereas the body weight-normalized heart weight did not differ significantly between these two groups (P>0.05; Fig. 1C), suggesting that cardiac hypertrophy is absent in the DOX-treated mice. Gene expression analyses of pooled heart samples revealed that the mRNA level of *Ctgf* (which encodes a pro-fibrotic cytokine) was significantly increased in DOX-treated mice compared to control animals (P<0.01; Fig. 1D). These findings suggested that cardiac tissue is the primary target of DOX cardiotoxicity. To further clarify the effects of DOX on the heart, we measured the serum levels of specific biomarkers for myocardial injury (including CK-MB and troponin T) as well as the serum levels of more general markers for inflammation (AST and LDH) (Fig. 2A-D). Mice that received DOX showed higher levels of not only AST and LDH, but also CK-MB and troponin T, as compared to control mice, supporting the use of the specific biomarkers as indicators of DICT. We also measured the mRNA levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in blood and heart, other biomarkers, at the first screening. Unfortunately, there was poor reproducibility in the measurements, so we did not adopt the data regarding natriuretic peptides.

Because DOX also can lead to hematotoxicity (30), some hematological parameters were measured in the small portion of whole blood collected from mice of both groups. In the DOX-treated mice, WBC values decreased to 45.8% of those in control mice, while platelet counts increased to 115.7% of those in control mice. Nevertheless, post-dose values of other parameters, such as RBC, Hb, and Hct, in DOX-treated mice did not differ significantly from those values in control mice (P>0.05) (data not shown), suggesting that DOX did not cause anemia or hypoglobulinemia.

Correlation of DICT and mRNA expression in blood. We next examined the correlation between the severity of DICT and blood mRNA expression of genes prior to DOX administration. Among 54 DOX-treated mice, twelve animals (22.2%) had no significant change in serum levels of cardiac injury parameters (P>0.05), while apparent cardiotoxicity (as evidenced by increases in the levels of the parameters associated with cardiotoxicity) was observed in 42 mice (77.8%), including ten mice (18.5%) that were found dead. The death was considered due to severe heart failure, because survival rate has conventionally been used as an index of DICT (31-33). Most of surviving mice that escaped from lethal DICT would develop heart failure or cardiotoxicity. To facilitate correlation analysis, susceptibility to DOX-induced heart damage was scored as 0 to 8 based on separate changes in parameters (AST, LDH, CK-MB, and troponin T) in surviving mice as follows: 0, no change in either parameter (no damage detected); 1, increase in either AST or LDH=1 (such that increases in both yielded a value of 2); 3, increase in either CK-MB or troponin T=3 (such that increases in both yielded a value of 6); and 8, increases in all of four parameters. For example, if three parameters except for troponin T increased in a blood sample, its DICT score was determined as 5 (AST=1, LDH=1 and CK-MB=3). In addition, death was given a score of 10 as a maximum cardiac injury.

As a first screen for mRNA expression, we used RT-qPCR to assess the blood levels of transcripts from 32 candidate genes, including those encoding apoptosis-, autophagy- and oxidative stress-related proteins, each of which might contribute to the pathological mechanism(s) of DICT. The results revealed that the susceptibility to DOX-induced heart damage (as described above) was significantly and positively correlated with blood mRNA levels of *Il6* (P<0.01) and *Pdcd1* (P<0.05) (Fig. 3), as well as with the blood mRNA levels of the genes encoding *Sod3* (P<0.01), *Gpx3* (P<0.05) and *Mt1* (P<0.05) (Fig. 4) prior to drug administration, suggesting that the products of these five genes may have some role in DICT. We chose to focus on the roles of *Il6* and *Pdcd1*, given that

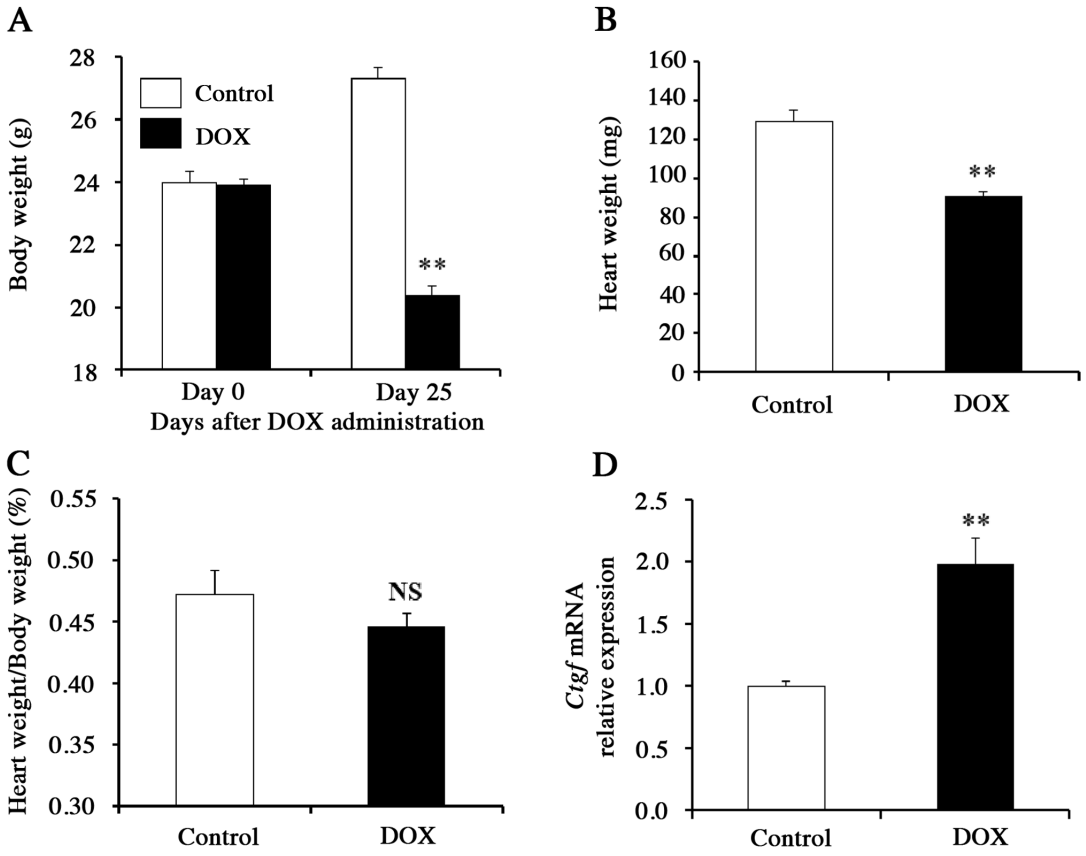

Figure 1. Changes in body weight, heart weight and cardiac expression of *Ctgf* mRNA in mice after cumulative administration of DOX. At 7 days after administration of the last dose (25 days after the initiation of DOX administration), (A) body weight, (B) heart weight and (C) body weight-normalized heart weight were determined, and (D) the mRNA level of *Ctgf* in heart tissue was measured by reverse-transcription quantitative PCR analysis. Each value is expressed as the mean ± SEM (n=18 for control mice, n=44 for DOX-treated mice). **P<0.01 vs. control mice. DOX, doxorubicin; *Ctgf*, connective tissue growth factor.

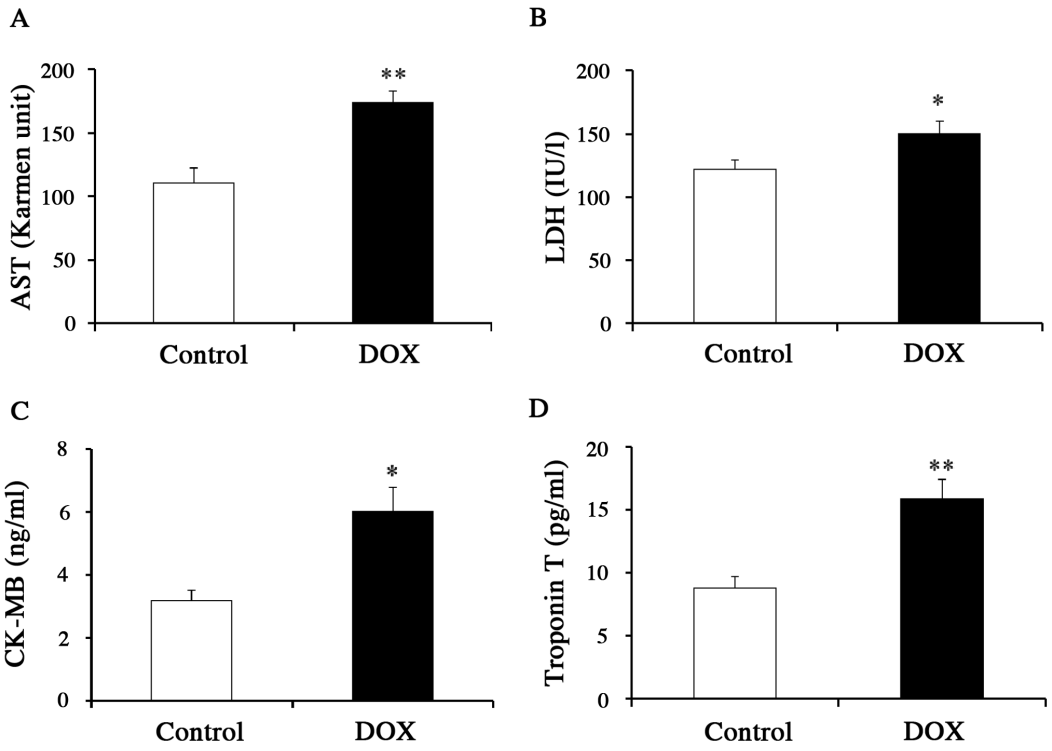

Figure 2. Changes in tissue injury parameters induced by cumulative administration of DOX. Mice were injected intraperitoneally with either 3.33 mg/kg DOX (a cumulative dose of 30 mg/kg) or an equivalent volume of saline every other day for 18 days. At 7 days after administration of the last dose, the serum levels of (A) AST, (B) LDH, (C) CK-MB and (D) troponin T were measured. Each value is expressed as the mean ± SEM. *P<0.05, **P<0.01 vs. control mice. AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK-MB, creatine kinase MB isoenzyme; DOX, doxorubicin.

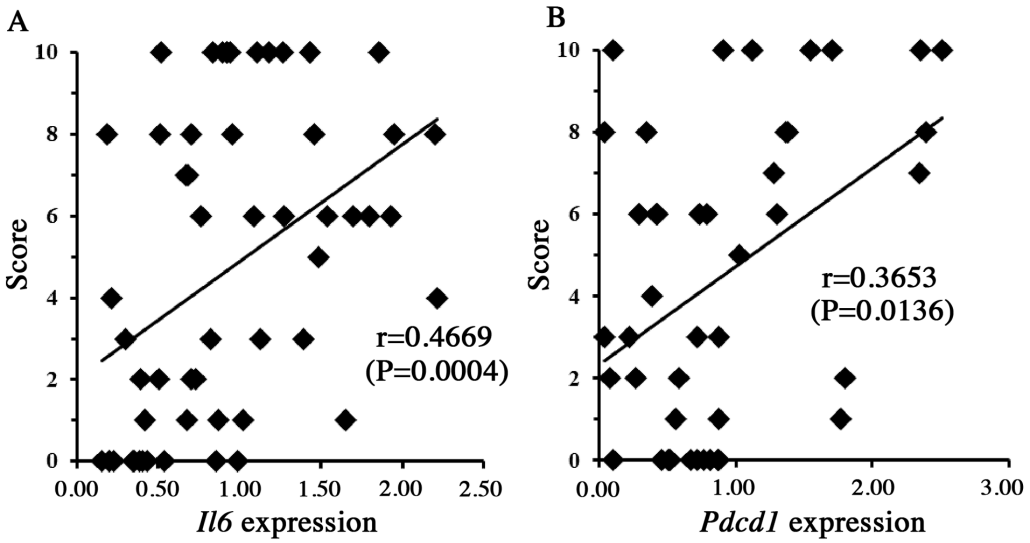

Figure 3. Correlation between the severity of cardiotoxicity after DOX administration and blood mRNA expression of *Il6* (A) or *Pdcd1* (B) before DOX administration. The severity of DOX-induced cardiotoxicity was scored by the changes in tissue injury parameters. The correlation in individual mice was determined by the Spearman correlation function and regression analysis. The correlation coefficient and statistical significance are presented in each figure. DOX, doxorubicin; *Pdcd1*, programmed cell death 1.

previous work has suggested that cell signaling mediated by IL-6 and PDCD1 contributes to cytoprotection in the heart and other tissues (34-39). Therefore, *in vitro* experiments were performed to clarify the roles of *Il6* and *Pdcd1* in DICT.

Effects of Il6 or Pdcd1 knockdown on DOX-induced apoptosis in vitro. We next assessed the potential roles of *Il6* and *Pdcd1* on DOX-induced toxicity of H9c2 cells (embryonic rat cardiac myoblasts), a line that is commonly used as a model for studies of DICT. Because cell death by apoptosis is involved in the primary mechanism of DICT (1,2,5), we studied effects of knockdown of *Il6* or *Pdcd1* on apoptotic response to DOX. The knockdown of endogenous *Il6* and *Pdcd1* was achieved by transfection with gene-specific siRNAs (Fig. S1). Knockdown efficacy was estimated by RT-qPCR; mRNA expression of *Il6* and *Pdcd1* were 0.32±0.03 and 0.23±0.05, respectively, when expression following transfection with the control siRNA was defined as 1.00 (Fig. S1A and B). Mock cells (vehicle control cells) exhibited no significant change in the accumulation of either mRNA (P>0.05). The knockdown of *Il6* and *Pdcd1* was confirmed by assessing levels of the two proteins by immunofluorescence analysis (Fig. S1C and D).

In mock cells exposed to DOX (1 μM), apoptosis (as determined by a luminescence assay) increased with incubation time. The DOX-induced apoptosis, however, was significantly increased in *Il6* knockdown cells (P<0.01) and in *Pdcd1* knockdown cells (P<0.05) than in mock cells within the first 10 h of incubation (Fig. 5A and B). Thus, DOX-induced apoptosis during the first 10 h of exposure was significantly enhanced by either *Il6* or *Pdcd1* knockdown, although the effects were weakened or undetectable for later time points (Fig. 5A-C). The activity of caspase-3/7, a protease enzyme that plays an essential role in apoptotic processes, also was increased in a DOX concentration- and exposure time-dependent manner in mock cells exposed to DOX (0.1-1 μM), confirming the ability of the corresponding assay to detect DOX-induced apoptosis. The increase in activity, however, was potentiated in each of the

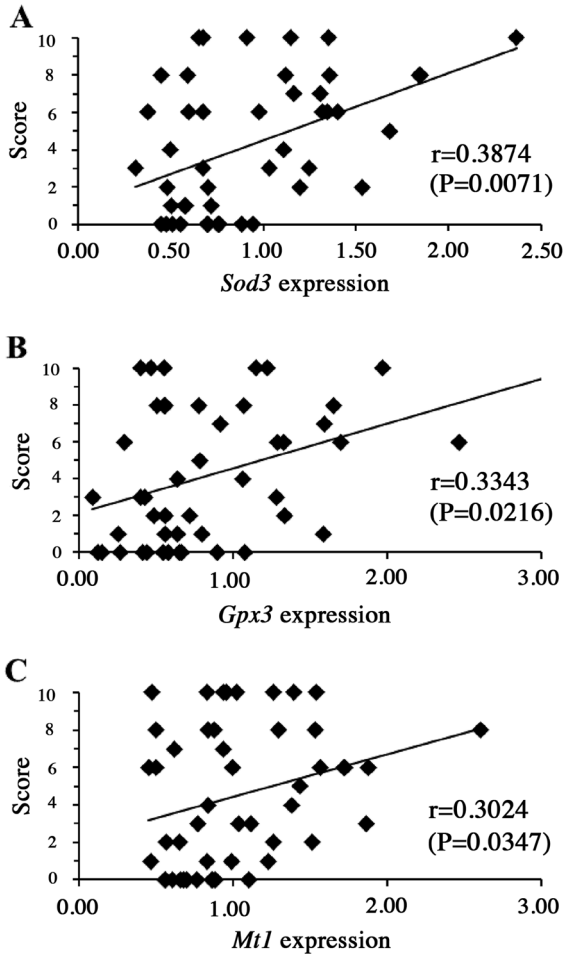

Figure 4. Correlation between the severity of cardiotoxicity after DOX administration and blood mRNA expression of *Sod3* (A), *Gpx3* (B) or *Mt1* (C) before DOX administration. The severity of DOX-induced cardiotoxicity was scored by the changes in tissue injury parameters. The correlation in individual mice was determined by the Spearman correlation function and regression analysis. The correlation coefficient and statistical significance are presented in each figure. DOX, doxorubicin; *Sod3*, superoxide dismutase-3; *Gpx3*, glutathione peroxidase-3; *Mt1*, metallothionein-1.

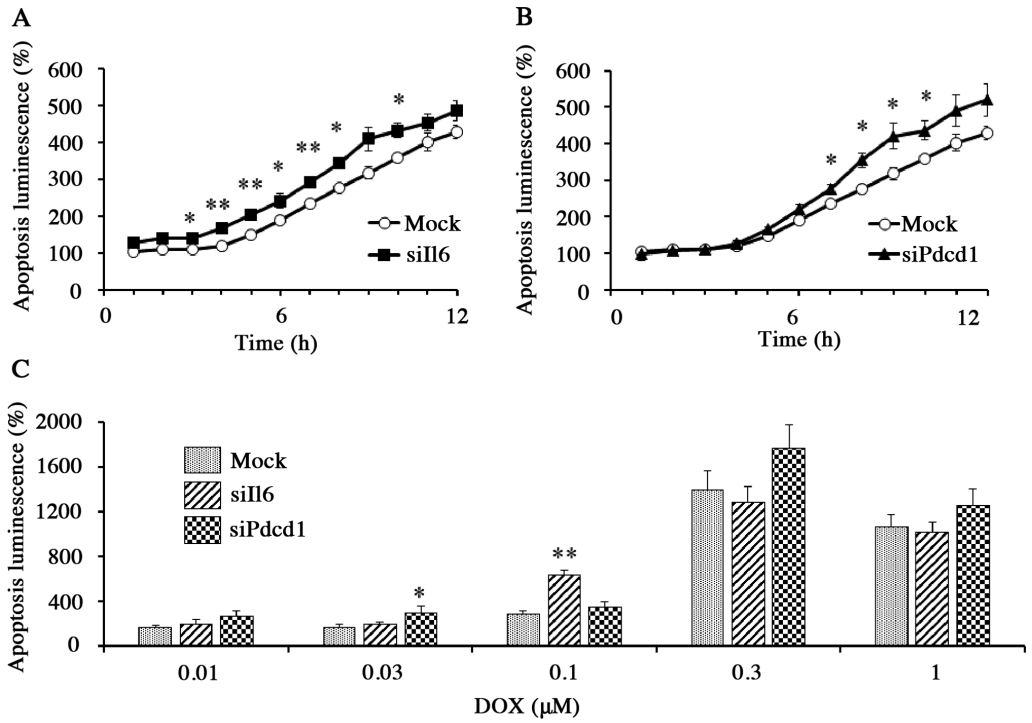

Figure 5. Effects of (A) *Il6* and (B) *Pdcd1* knockdown on DOX-induced apoptosis in H9c2 cells. Apoptosis was determined by a luciferase assay using annexin V, an apoptosis-specific marker; the degree of apoptosis in DOX-treated cells is expressed as a percentage of that in control (no DOX exposure) cells. Apoptosis in (A) *Il6* and (B) *Pdcd1* knockdown cells was evaluated after incubation with DOX (1 μM) for various time periods (1-12 h). The mock group presented in (A) and (B) is not the same as independent group. (C) Similar evaluation of apoptosis was performed after incubation for 24 h with various concentrations of DOX (0.01-1 μM). Each value is expressed as the mean ± SEM of three samples. *P<0.05, **P<0.01 vs. mock cells. DOX, doxorubicin; *Pdcd1*, programmed cell death 1; si, small interfering.

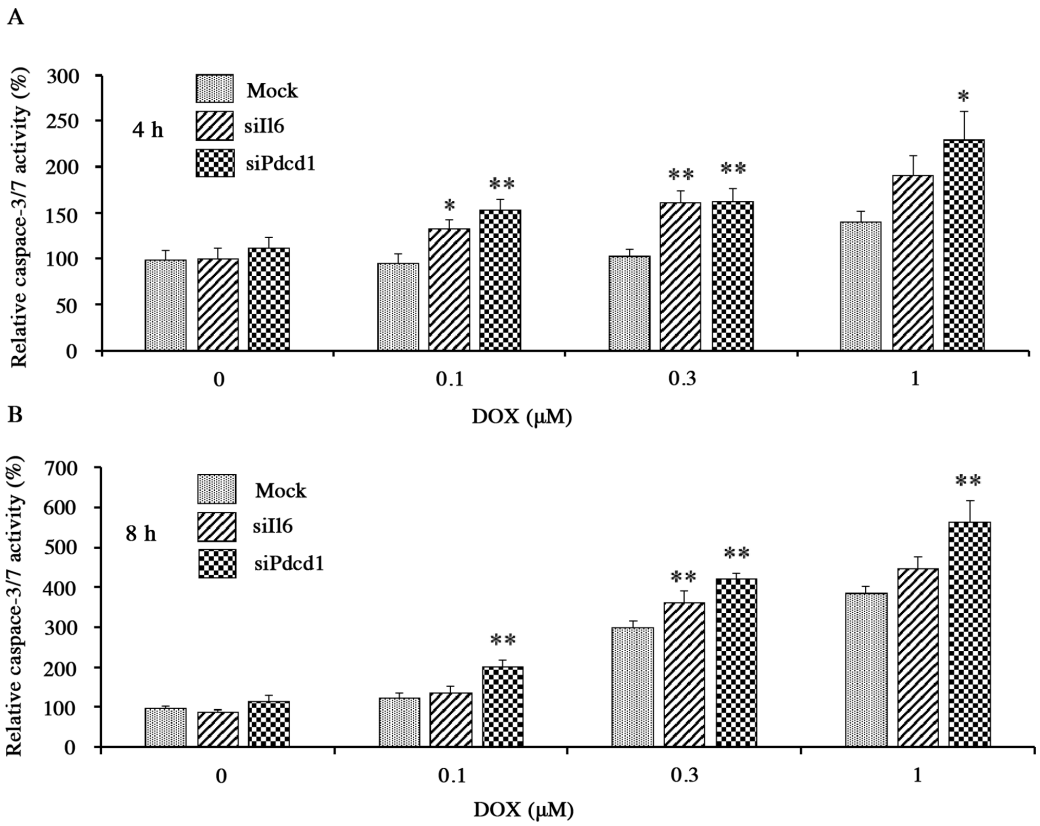

Figure 6. Effects of (A) *Il6* and (B) *Pdcd1* knockdown on DOX-induced changes in caspase-3/7 activity, a marker of apoptosis, in H9c2 cells. After cells were incubated for (A) 4 h or (B) 8 h with DOX (at 0.1, 0.3 and 1 μM), caspase-3/7 activity was assessed by luciferase assay. The degree of caspase-3/7 activity is expressed as a percentage of DOX-untreated control cells. Each value is expressed as the mean ± SEM of three samples. *P<0.05, **P<0.01 vs. mock cells. DOX, doxorubicin; *Pdcd1*, programmed cell death 1.

knockdown cells compared to the mock cells (Fig. 6A and B); for example, after incubation for 4 h with 0.1 μM DOX, activities in *Il6* and *Pdcd1* knockdown cells were elevated by ~30 and ~60%, respectively, compared to mock cells (Fig. 6A). Thus, by two separate assays, DOX-induced apoptosis was enhanced by knockdown of *Il6* or *Pdcd1*, indicating that these genes have a preventive role against DICT.

Discussion

DICT can result in acute or chronic adverse effects. Both acute and chronic toxicity may lead to cardiac dysfunction or cardiomyopathy, eventually leading to severe heart failure and death (40,41). While acute cardiotoxicity following treatment with a high dose of DOX is now rare (40), late-onset chronic cardiotoxicity induced by the cumulative DOX dose remains common, occurring in up to 65% of patients (42). In the present study, we used a chronic cardiotoxicity model, in which DICT was established in mouse by repeated administration of DOX.

DOX-treated mice showed elevations (compared to control mice) in serum levels of CK-MB and troponin T, specific markers of myocardial cell injury, as well as in serum levels of non-specific cytosolic enzymes, such as AST and LDH. Although we did not assess the early cardiotoxicity markers after DOX administration, the data on early markers may help to find new information and knowledge about DICT. We need consider these points in our next study. The mice also had elevated (compared to control mice) cardiac tissue levels of the *Ctgf* transcript, which is associated with fibrosis after heart tissue damage. These biochemical changes indicate that DOX induces lethal injury in myocardial cells. It should be noted, however, that no myocardial injury was found in a subset (22.2%) of DOX-treated mice, although the majority of mice displayed apparent DICT, including death. Thus, individual differences are likely to exist in sensitivity of the heart to DOX. Clinical findings also have indicated that individuals vary greatly in their sensitivity to DOX (5-7); for instance, some patients are highly tolerant to DOX at cumulative doses exceeding 1,000 mg/m^2, while others exhibit cardiotoxicity at cumulative doses below 400 mg/m^2 (5).

It is possible that differences among individuals are attributable to genetic factors, which can be associated with the pathogenesis of DICT. To assess this possibility, we analyzed the correlation between mRNA expression of candidate genes in blood before DOX administration and the severity of DICT (using a score based on the changes in parameters known to be indicative of cardiac injury). Among 32 candidate genes, we have identified the five genes (*Il6, Pdcd1, Sod3, Gpx3* and *Mt1*) that were correlated with susceptibility to DICT. According to previous reports (34-39), cell signaling mediated by IL-6 and PDCD1 may be involved in protection from myocardial damage, such as ischemic damage, and therefore we investigated the roles of *Il6* and *Pdcd1* in DICT. As shown in Fig. 3, higher mRNA expression of *Il6* or *Pdcd1* was positively correlated with the DICT score, indicating that the expression levels of these genes might serve as predictive markers for DICT. In addition, these results led to the expectation that the expression of *Il6* or *Pdcd1* (at the mRNA or protein level) may be factors influencing the development of DICT. In the next experiment, therefore, DOX toxicity was assessed in cells of the rat cardiomyocyte line H9c2 in which *Il6* or *Pdcd1* expression had been subjected to knockdown via transfection with gene-specific siRNAs (Fig. S1). The cell toxicity of DOX was evaluated by monitoring apoptosis, a process known to be one of the primary mechanisms of DICT (1,2,5). Apoptosis was assessed by an annexin V assay, previously demonstrated to show high sensitivity and specificity for cell death (26), and by a caspase-3/7 assay known to be indicative of apoptotic processes (28). Contrary to our expectation, cells subjected to *Il6* and *Pdcd1* knockdown exhibited increased (rather than decreased) levels of DOX-induced apoptosis, suggesting that both of these genes play a protective role against the DOX-induced cardiomyocyte apoptosis, at least in this rat cardiomyocyte line.

IL-6 has been shown to be a pro-inflammatory cytokine with cardioprotective potential (35). Indeed, many reports have stated that the expression of this cytokine in cardiomyocytes attenuates myocardial ischemia-reperfusion damage (34,35,39). However, consistent with our results for blood concentrations of *Il6* transcripts in mice, the circulating levels of IL-6 have been shown to be elevated in patients with congestive heart failure (43), and IL-6 is known to be released from the border zone of myocardial infarcts (44). The increased circulating levels of *Il6* transcript and protein may reflect the release of these gene products into blood as the result of a compensatory mechanism(s) intended to reduce the vulnerability of heart tissue to various stresses such as ischemia and chemical stimuli, leading to cardioprotective action. In contrast, IL-6 has also been found to act as a deleterious cytokine associated with oxidative stress on the heart. The cytokine is indicated to induce apoptosis in cardiomyocytes (45), in agreement with our findings in terms of role of IL-6 on apoptosis. Furthermore, involvement of IL-6 (and oxidative stress) in the pathogenesis of cardiac heart failure (46) and atrial fibrillation (47) has been reported. The cardiac role of IL-6 is complex and remains poorly understood (48).

PDCD1, an immune inhibitory receptor, has been shown to inhibit lymphocyte activation and cytokine production (49). A previous study in mouse demonstrated that neutralization of PDCD1 with an anti-PDCD1 monoclonal antibody enhances DOX-induced nephropathy, suggesting that the PDCD1 pathway protects renal tissue from DOX-associated toxicity (38). That finding would be in agreement with our results suggesting the beneficial action of PDCD1 in preventing DOX-induced cardiotoxicity, although the underlying mechanism(s) of this effect remain unclear. Further studies will be needed to determine the details of the pathways and mechanisms whereby IL-6 and PDCD1 counteract DOX toxicity.

Extensive efforts have been made in various model systems to understand the role of gene expression in the mechanism(s) of DICT pathogenesis (21,50-55). These studies assessed early and time-dependent molecular changes that occur following DOX administration, but did not include assessment of the levels of the tested molecules prior to DOX administration. For instance, troponins, which are sensitive tissue-specific markers of heart damage, are known to accumulate to elevated levels in blood only after cardiac tissue damage has occurred (56), but (to our knowledge) the pre-exposure levels of troponins have not previously been investigated in the context of DOX exposure. Invasive and noninvasive clinical approaches are

currently being tested for use in predicting cardiotoxicity in DOX-treated cancer patients, but there have been technical complications (57,58). In that context, the results obtained in the present study may contribute to the early prediction of DICT and to new therapeutic strategies of cardioprotection. To our knowledge, the present report is the first to demonstrate a role for *Il6* and *Pdcd1* as predictive and protective factors in DICT. If animals with diverse genetic backgrounds are used, further genes associated with DICT may be found. Nevertheless, we used C57BL/6J inbred mice to minimize the individual differences including age, body weight, physical condition and genetic background, all of which could significantly influence the DICT. Although it is difficult to apply our findings to a human population in terms of genetic diversity, we believe that *Il6* and *Pdcd1* may also provide a beneficial role in cardioprotection in clinical chemotherapy with DOX.

Pharmacological strategies for preventing DICT have also been proposed in many studies; β-blockers, ACE inhibitors, ARBs and statins can reduce DICT in animals and in humans (1,10,13). It is noteworthy that dexrazoxane, which reduces oxidative stress (by iron chelation) and inhibits topoisomerase IIβ, protects the heart from anthracycline-induced toxicity (21,59,60). In fact, dexrazoxane is licensed in many parts of the world for two different indications; prevention of cardiotoxicity from anthracycline-based chemotherapy, and prevention of tissue injuries after extravasation of anthracycline (60). Based on information on predictive gene expression in individual patients, a pharmacological approach may be useful for further effective protection against DICT.

In conclusion, the pre-existing level of expression of both *Il6* and *Pdcd1* in cardiomyocytes may play an important role in protection against DOX-induced damage, such that the expression of these genes in blood serves as a predictive marker for DICT. These findings may provide useful information for prevention of cardiotoxicity in cancer patients receiving DOX therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SK performed the experimental work, data collection and writing of the paper. AH participated in the study design, interpreted the data and revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experiments were conducted in accordance with the standards established by the Japanese Pharmacological Society and were approved by the Tohoku Medical and Pharmaceutical University of Institutional Animal Care and Use Committee (Experimental Protocol no. 18013).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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