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Recombinant human interleukin-1 receptor antagonist protects mice against acute doxorubicin-induced cardiotoxicity

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ABSTRACT

Doxorubicin is a potent anticancer drug which is widely used in the treatments of a variety of solid and hematopoietic tumors, but its use is limited by its cardiotoxicity and dose-dependent congestive heart failure. After finding a close connection between Interleukin-1 family and doxorubicin-induced cardiotoxicity, we assumed that recombinant human interleukin-1 receptor antagonist (rhIL-1Ra), the natural antagonist of interleukin-1, might have a protective role in doxorubicin-induced cardiotoxicity. In this report, Balb/c mice were intraperitoneally injected with doxorubicin (18 mg/kg) followed by injections of 1 mg/kg rhIL-1Ra 4 h later, and consecutive daily injections of rhIL-1Ra on the following 4 days (1 mg/kg/day). We found that rhIL-1Ra significantly decreased malondialdehyde in cardiac tissue and prevented doxorubicin-associated cardiac troponin I elevations in serum, especially at day 14 after doxorubicin treatment. Importantly, rhIL-1Ra diminished doxorubicin-induced microstructural damages of cardiac tissue and rescued doxorubicin-caused reduction of cardiac functions exemplified by ejection fraction and fraction shortening. Our results reveal a potential role of rhIL-1Ra in protecting mice against doxorubicin-induced cardiac injuries and lead to a conclusion that this protein may be a potential candidate agent that inhibits cardiomyocyte-toxicity in doxorubicin-exposed patients.

1. Introduction

Doxorubicin (DOX) is a potent anticancer drug that is widely used clinically in treating leukemia, lymphomas and various solid tumors of the lung and breast (Young et al., 1981). However, DOX in cancer treatment is limited because of its cardiotoxicity (Torti et al., 1986; Horan et al., 2006) and dose-dependent congestive heart failure, which was proved to be often presenting several years after cessation of treatment (Singal and Iliskovic, 1998; Steinherz et al., 1991). Intensive studies demonstrate that the mediating mechanism for this cardiotoxic effect is primarily via the generation of oxidative stress. The O^2- which is generated by DOX via a redox cycling reaction can give rise to a variety of more active reactive oxygen species, including H_2O_2, UOH and ONOO–, which trigger further oxidations of biomolecules (Chen et al., 2006) and lead to tissue-specific mitochondrial damages (Berthiaume and Wallace, 2007), disturbances of calcium (Kusuoka et al., 1991; Temma et al., 1997) or iron (Minotti et al., 1999).

Inflammatory cytokines contribute to heart disease progression on myocardial function as well as structural remodeling (Willerson and Ridker, 2004; Mann, 2002). Among them, Interleukin-1 (IL-1) plays critical roles in the pathogenesis of heart disease (Bujak and Frangogiannis, 2009). IL-1 binds the transmembrane IL-1 type I receptor (IL-1R1) and initiates down-stream signals. Its natural antagonist, IL-1 receptor antagonist (IL-1ra), competitively interacts with the same receptor to prevent IL-1 signaling (Dinarello, 1996). IL-1Ra via local overexpression in heart was shown to be cardioprotective in rat myocardial ischemia–reperfusion injury and mouse viral myocarditis (Suzuki et al., 2001; Lim et al., 2002). Recently, IL-1Ra in its recombinant form (Anakinra) was shown to inhibit cardiomyocyte apoptosis in a rat model of acute myocardial infarction (Abbate et al., 2008). Furthermore, Anakinra improved vascular and left ventricular function in patients with rheumatoid arthritis but without coronary artery disease (Ikonomidis et al., 2008).

Given the importance of IL-1 signaling in heart diseases and the unmet well-defined medical problem of DOX-induced cardiotoxicity, we provide for the first time evidence that IL-1 participates in the

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disease progress of DOX-induced cardiotoxicity, and the administration of IL-1Ra has beneficial effects in DOX-treated mice.

2. Material and methods

2.1. Animals

The pathogen-free, sex-matched, 8-week-old Balb/c mice (SLAC-CAS, Shanghai, China) were maintained in air-filtered units at 21 °C ± 2 °C and 50% ± 15% relative humidity throughout the experiment. Mice were fed with sterile water and rodent food. Animal experiments were performed with the authorization of Animal Care and Use Committee of School of Pharmacy of Shanghai Jiaotong University.

2.2. Study design

First, in experiment 1, in order to find the connections between IL-1 family and DOX-induced cardiotoxicity, mice were randomly divided into 4 groups. Group 1 was treated with intraperitoneal injection of saline as control; group 2 was treated with intraperitoneal injection of 12 mg/kg DOX (Hisun Pharmaceutical, Zhejiang, China); 15 mg/kg and 18 mg/kg DOX were administrated in groups 3 and 4, respectively. The animals were killed at day 7 post-DOX administration and the hearts were removed. Heart tissues were homogenated for RT-PCR and western blot analysis of IL-1 family. Meanwhile, serum concentrations of IL-1β and IL-1Ra were measured.

Then, in experiment 2, in order to find the exact effects of rhIL-1Ra in DOX-induced cardiotoxicity, mice were randomly divided into 2 groups. Group 1 was treated with intraperitoneal injection of DOX (18 mg/kg) followed by injections of rhIL-1Ra (1 mg/kg) 4 h later, and consecutive daily injections of rhIL-1Ra on the following 4 days (1 mg/kg/day); the same volume of saline was used instead of rhIL-1Ra in group 2. RhIL-1Ra was purified with the method previously described for recombinant murine interleukin-1 receptor antagonist purification (Zhang et al., 2008) with minor modifications. Further characterizations were performed after the treatments.

2.3. IL-1β and IL-1Ra ELISA

Mice peripheral blood samples were obtained by retro-orbital puncture. Blood samples were coagulated after stored in the refrigerator at 4 °C overnight. After centrifugation at 1000×g for 15 min, the serum was collected and stored at −80 °C. Aliquots were used once only for the test and were not subjected to repeated freeze–thaw cycles. Murine IL-1β and IL-1Ra ELISA kits (R&D, Minnesota, USA) were used to measure IL-1β and IL-1Ra serum levels according to the instructions of the manufacturer.

2.4. RT-PCR analysis of IL-1 family

Heart tissues in each group were removed and total RNA was extracted using TRIzol Reagent (Invitrogen, California, USA) according to the manufacturer's protocol. The expression of IL-1 family mRNAs, including IL-1a, IL-1β, IL-1RI, IL-1RII, IL-1Ra, was analyzed by RT-PCR, using specific primers according to Gabellec et al. (1997) report. 4 μl of the PCR products was analyzed by electrophoresis on a 1% agarose gel, followed by ethidium bromide staining to determine the levels of IL-1 family mRNAs relative to the control GAPDH transcript.

2.5. Western blot analysis of IL-1RI

To analyze expression of IL-1RI protein, heart tissues were pooled for each experimental condition and homogenized. Equal amount of protein sample (80 μg) was separated by SDS-PAGE and electrotransferred to PVDF membranes. IL-1RI was probed with Rabbit anti-mouse 1:250 dilution (Abcam, Massachusetts, USA) for 1 h, followed by extensive washes with Tris Buffered Saline supplemented with 0.1% Tween-20. After hybridized with goat radish-peroxidase-conjugated secondary antibody at a dilution of 1:1000 for 1 h, the immunoreactive proteins were detected using an ECL western blotting detection system (Beyotime Institute of Biotechnology, Jiangsu, China). Detection of GAPDH was used as the protein loading control.

2.6. Serum cardiac troponin I analysis

The activity of serum cardiac troponin I has been widely used clinically as a parameter for the diagnosis of cardiac diseases and it is also proved to be a sensitive, specific biomarker of cardiac injury in laboratory animals (O'Brien et al., 2006). Serum cardiac troponin I was analyzed with the method of ELISA by Access AccuTnI (Beckman, California, USA).

2.7. Malondialdehyde content test

Malondialdehyde is a marker of lipid peroxidation and free radical activity, as reactive oxygen species degrade polyunsaturated lipids and form malondialdehyde (Del Rio et al., 2005). Cardiac tissue samples were homogenized in 10% (w/v) Tris-buffered saline (Ph7.4, 0.01 mol/L Tris–HCl, 0.0001 mol/L EDTA-2Na, 0.01 mol/L sucrose, 0.8% NaCl) at 4 °C with a homogenizer. Homogenates were then centrifuged at 500×g for 15 min. The supernatants were collected and evaluated using the malondialdehyde assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

2.8. Pathologic examination

Cardiac tissue samples were fixed in 10% formalin, dehydrated through an upgraded ethanol series and embedded in paraffin blocks. Ultrathin sections of 5 μm were dewaxed by xylene, hydrated through a degraded ethanol series, and stained with haematoxylin and eosin. A pathologist blinded to the treatments performed the pathologic examination under an optical microscopy (×400). Sections were assigned grades as reported by Bertinchant et al. (2003) with minor modifications. The findings are classified as: perivascular fibrosis, interstitial fibrosis, myocyte vacuolisation and degeneration. The severity of the findings is graded: 0, no changes; 0.5, very slight; 1, slight; 2, moderate; 3, marked. 9 heart sections per mouse were analyzed for each experimental time point.

2.9. Echocardiography

Echocardiography is widely used clinically for detecting patients' cardiac function. To determine whether rhIL-1Ra treatment had any effect on mice's cardiac function, we performed M-mode echocardiography with the small animal echocardiography analysis system of Vevo 770 (Visualsonics Inc., Toronto, Canada). Briefly, mice were lightly anesthetized by inhalation of 1–1.5% isoflurane, and the upper sternal and subxiphoid areas were shaved and then moistened for better acoustic coupling. Electrode was attached to mice limbs and parameters of the cardiac cycle were recorded by M-mode echocardiography of the left ventricle at the tip of the mitral valve apparatus with a 30 MHz transducer. To describe the function of the left ventricles, ejection fraction and fraction shortening were calculated.

2.10. TUNEL assay

The slides of heart tissues were processed for the terminal dUTP nick end-labeling (TUNEL) assay. An ApopTag in situ detection kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Briefly, the slides were treated with H_2O_2 and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-conjugated dUTP for 1 h

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at 37 °C. Labeled DNA was visualized using a fluorescence microscope. For negative control, terminal deoxynucleotidyl transferase was omitted from the reaction mixture. The percentage of TUNEL-positive cells was determined by counting at least 1000 cells in 5 randomly selected fields.

2.11. Statistics

Results are expressed as mean ± standard deviation (S.D.). Statistically significant differences over time in the same treatment group, or among different treatment groups at a single time point were determined by one-way analysis of variance (ANOVA), followed by two-tailed Student's t-tests. Results from survival experiments were analyzed using the log-rank test. Statistical significance was assumed for $P<0.05$.

3. Results

3.1. IL-1 family is closely connected with DOX-induced cardiotoxicity

In experiment 1, the expressions of IL-1β and IL-1Ra in serum were both highly induced on day 7 ($P<0.01$) after different dosages of DOX treatments, especially in the highest dosage group (18 mg/kg) (Fig. 1A, B). RT-PCR analysis showed upregulations of IL-1β, IL-1RI, IL-1RII and downregulation of IL-1a in all of the groups with DOX treatment, especially in the highest dosage group, while the expression of IL-1Ra was found not to be changed. GAPDH was used as the loading control (Fig. 1C). Western blot analysis of protein extracts from cardiac tissue also confirmed the RT-PCR analysis results. Quantitative measurement of western blot images as relative IL-1RI protein compared to GAPDH showed that IL-1RI was strongly expressed in cardiac tissue on day 7 after DOX treatment (Fig. 1D).

3.2. RhIL-1Ra protects mice against DOX-induced body weight loss and mortality

Eight days after a single injection of 18 mg/kg DOX, 53.3% (16 out of 30) mice died in the DOX + saline group, while in the DOX + rhIL-1Ra group only 36.7% (11 out of 30) mice died. All survived mice in two groups appeared weak, but the administration of DOX + saline led to significant decrease in body weight compared to the DOX + rhIL-1Ra group (Table 1).

3.3. RhIL-1Ra prevents DOX-associated cardiac troponin I elevation in serum

Level of serum cardiac troponin I was measured in order to characterize the cardioprotective effects of rhIL-1Ra. DOX treatment had markedly increased serum cardiac troponin I concentrations, but

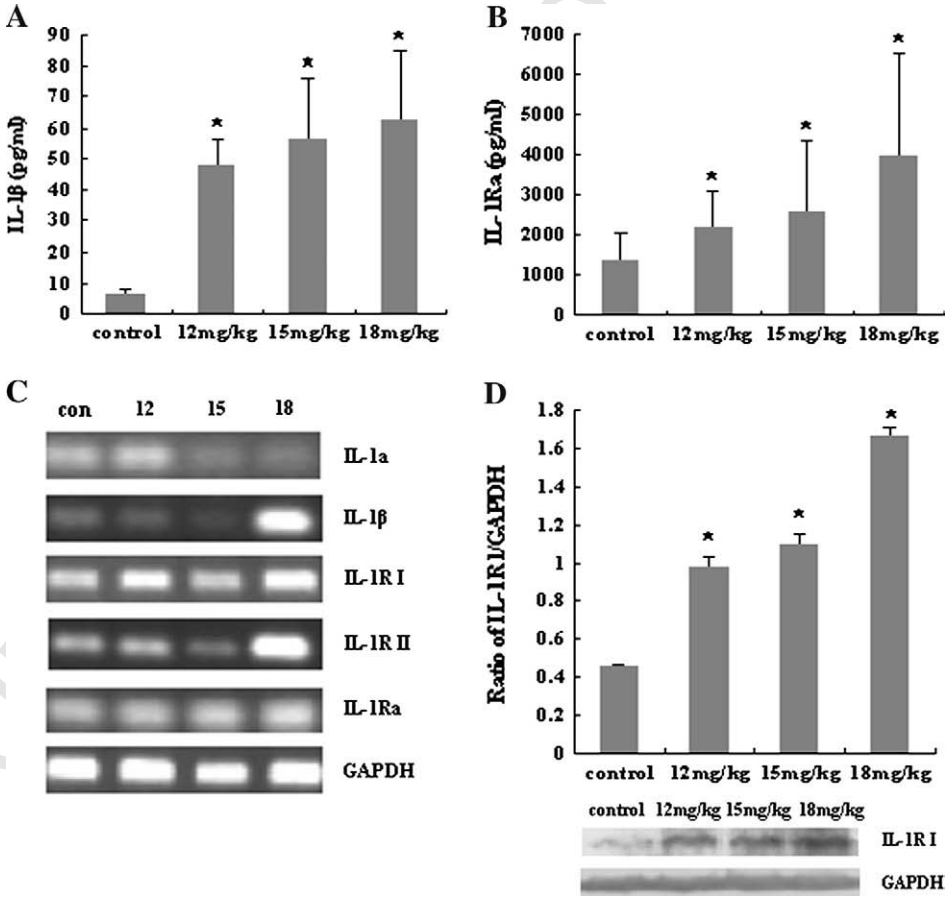

Fig. 1. IL-1 family expression in DOX-induced cardiotoxicity. (A, B) Serum samples obtained from peripheral blood were assayed for IL-1β and IL-1Ra by ELISA. Serum samples of 5 mice per group were analyzed individually, significant increase was detected on day 7 after all different dosage of DOX treatment, especially in the highest dosage group (18 mg/kg), compared to the pretreatment level (control). (C) RT-PCR analysis showed significant upregulation of IL-1β, IL-1RI, IL-1RII and downregulation of IL-1a after DOX treatment, compared to the control group, especially in the 18 mg/kg DOX group too, but no different expression of IL-1Ra was observed in each group. GAPDH was used as the loading control. (D) Western blot analysis of myocardial protein using antibodies against IL-1RI and GAPDH confirmed the RT-PCR analysis result, respectively. With three heart tissues mixed in each group, quantitative measurement of western blot images as relative IL-1RI protein compared to GAPDH showed IL-1RI was strongly expressed in cardiac tissues on day 7 after DOX treatment, compared to the pretreated cardiac tissue (control). All values are given as mean ± S.D. *Significantly different compared to the pretreated cardiac tissue (control) ($P<0.05$).

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Table 1
Effects of rhIL-1Ra on DOX-induced changes in body weight, heart weight and mortality of mice (8 days after DOX treatment).

Animal group	Body weight (g)	Heart weight (mg)	Heart/body weight ratio ($\times 10^3$)	Mortality
Control	24.0 ± 1.2	102.6 ± 0.3	4.31 ± 0.04	0
DOX + saline	17.8 ± 0.8[a]	69.0 ± 2.7[a]	3.92 ± 0.10[a]	53.3[a]
DOX + rhIL-1Ra	19.5 ± 1.7[a,b]	72.7 ± 1.5[a]	4.06 ± 0.08[a]	36.7[a,b]

For the mortality data there were 30 mice in each group. Other data were mean ± S.D. of 3–5 mice in each group.
[a] Significantly different from respective values in the control group ($P<0.05$).
[b] Significantly different from respective values in the DOX + saline group ($P<0.05$).

rhIL-1Ra treatment prevented most of these increases, especially on day 14 (Fig. 2A).

3.4. RhIL-1Ra protects cardiac tissue from malondialdehyde evaluation after DOX treatment

After 14 days of DOX treatment, there was a significant increase of malondialdehyde level in heart tissues in the DOX + saline group compared to the DOX + rhIL-1Ra group, indicating that rhIL-1Ra protects cardiac tissue from malondialdehyde evaluation after DOX treatment (Fig. 2B).

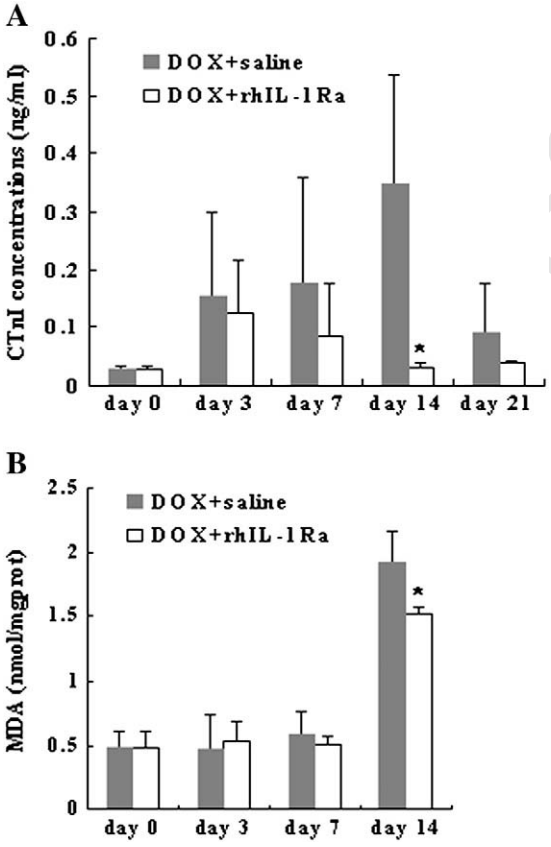

Fig. 2. RhIL-1Ra prevents DOX-associated cTnI and MDA elevations. (A) Serum cTnI concentrations on days after administration. Mean cTnI concentrations were significantly increased in each group after given DOX 18 mg/kg, but data in the DOX + rhIL-1Ra group was significantly lower than that of the DOX + saline group on day 14. (B) Treatment with rhIL-1Ra significantly decreased MDA level in heart homogenates on day 14 after DOX treatment (mean ± S.D. n = 3–5). cTnI, cardiac troponin I; MDA, malondialdehyde. *Significantly different compared to the DOX + saline treatment ($P<0.05$).

3.5. RhIL-1Ra prevents heart damage after DOX treatment

The cardiac tissue from control group (Fig. 3A) showed no remarkable pathological finding. However, 14 days after DOX treatment, cytoplasmic vacuolation and myofibrillar disorganization were prominent in the cardiac tissue from the DOX + saline group (Fig. 3B). In contrast, DOX-induced pathological changes were attenuated partially and focal myofibrillar loss was less in the DOX + rhIL-1Ra group (Fig. 3C). Grading by the method motioned above revealed that rhIL-1Ra significantly protects cardiac tissue from DOX injuries on day 14 after DOX treatment (Fig. 3D).

3.6. RhIL-1Ra protects mice against DOX-induced cardiac function loss

M-mode echocardiography analysis was performed with the small animal echocardiography analysis system. The stable beat rates of all animals in each group indicated the appropriate anaesthetic state during the operation (500–550 bpm). In order to measure the left ventricles in different groups, ejection fraction and fraction shortening were calculated. Prior to receiving any treatment, the mice did not show any difference in the basal values for both parameters (data not shown). After the treatments, DOX alone significantly decreased both ejection fraction and fraction shortening. In contrast, in the combined treatment of rhIL-1Ra and DOX, rhIL-1Ra completely rescued ejection fraction and fraction shortening (Fig. 4).

3.7. RhIL-1Ra prevents DOX-induced cardiomyocyte apoptosis

TUNEL-positive cells were observed significantly among the DOX + saline group after 14 days of DOX treatment, but slight in the DOX + rhIL-1Ra group. There were significant decrease of apoptosis cells in the DOX + rhIL-1Ra group after counting with Image-Pro Plus System (Roper Industries, California, USA), indicating that rhIL-1Ra can protect cardiomyocyte from DOX-induced apoptosis (Fig. 5).

4. Discussion

After finding the high expression of IL-1RI gene in mice cardiac tissues with DOX-induced cardiotoxicity in our previous study (data not shown), we assumed that IL-1 might participate in the process of DOX-induced cardiotoxicity. In order to confirm this hypothesis, we designed three different dosages of DOX treatment. RT-PCR analysis of gene and western blot analysis of protein both showed IL-1RI expressed highly in cardiac tissue after 7 days of DOX treatment, especially in the highest dosage (18 mg/kg) group. Furthermore, serum ELISA tests revealed that both IL-1β and IL-1Ra were highly induced in all DOX-treated mice. With these results, we hypothesized that IL-1β might increase the cardiac damage induced by DOX treatment and IL-1Ra could prevent this process.

We then investigated the effects of rhIL-1Ra in DOX-induced cardiotoxicity in wild-type Balb/c mice. With the subcutaneous injections of rhIL-1Ra in order to inhibit the activity of IL-1 on cardiomyocytes, we had confirmed that decreases of malondialdehyde in cardiac tissue and preventions of DOX-associated cardiac troponin I elevations in serum, especially on day 14 after DOX treatment, revealed the activities of rhIL-1Ra that leads to a protection against DOX-induced cardiotoxicity. Histopathological analysis and M-mode echocardiography analysis had also showed that rhIL-1Ra can prevent the heart damage and increase ejection fraction and fraction shortening after DOX treatment.

Our data have proved the high expression of IL-1β and indicated its potential damage in DOX-treated cardiotoxic mice, even though we don't know the exact mechanism. Previous studies suggested that there were no evidences proving that IL-1β was participating in the straight mechanism of DOX-induced cardiotoxicity, indicating

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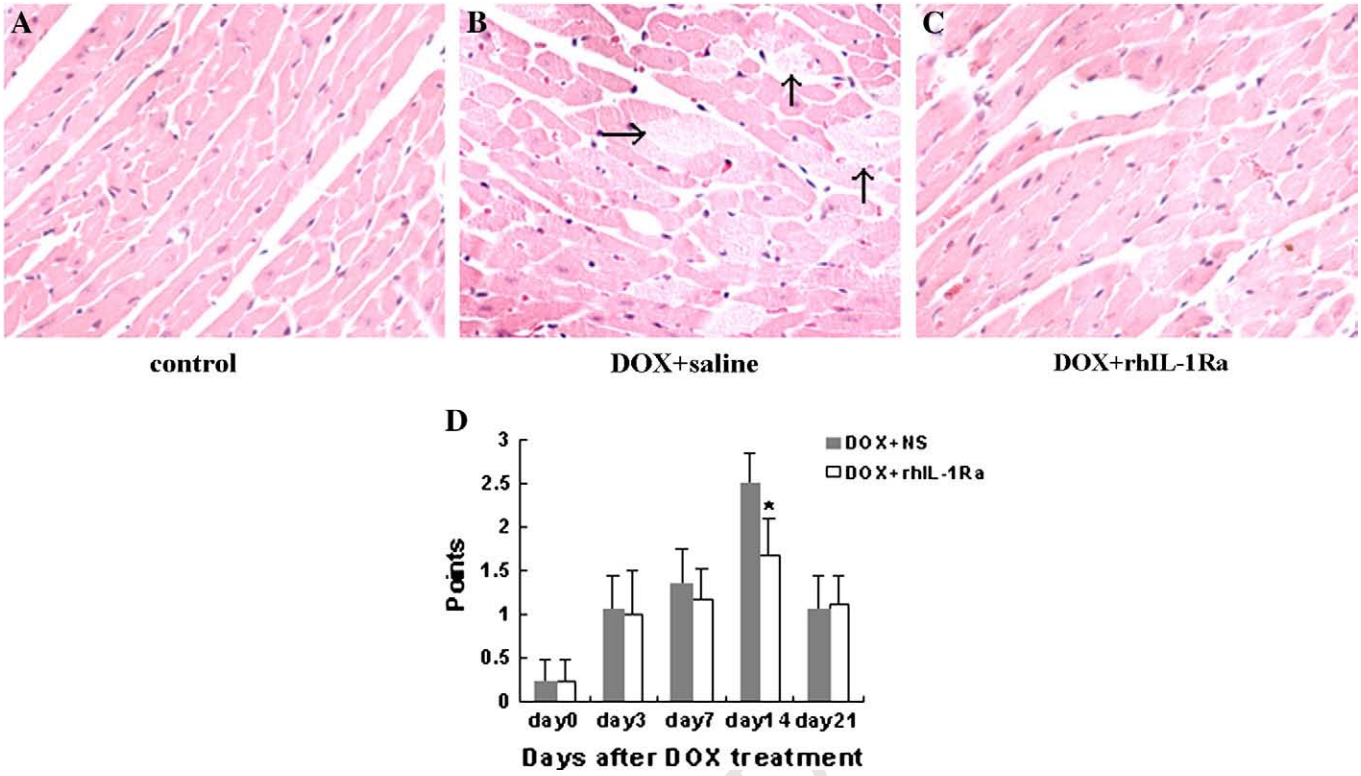

Fig. 3. RhIL-1Ra prevents heart damage after DOX treatment. (A, B, C) Light micrograph of cardiac tissue from control, day 14 after treated with DOX + saline and DOX + rhIL-1Ra (H&E × 400), showing cytoplasmic vacuolation and myofibrillar disorganization were prominent in the DOX + saline group (arrowhands), but less in the DOX + rhIL-1Ra group. (D) Grade of cardiac tissues showing that rhIL-1Ra significantly protects heart from DOX injuries on day 14 after DOX treatment (mean ± S.D. n = 9). *Significantly different compared to the DOX + saline treatment ($P<0.05$).

that IL-1β may increase cardiac damage via other ways. The discovery by Ing et al. (1999) provides some helpful insights. They had proved that IL-1β can induce neonatal cardiac myocyte apoptosis and this IL-1β toxicity results from the induction of NO. NO exerts its effect on tissues through several mechanisms, one of them is activating guanylate cyclase, which can react with oxygen free radicals to

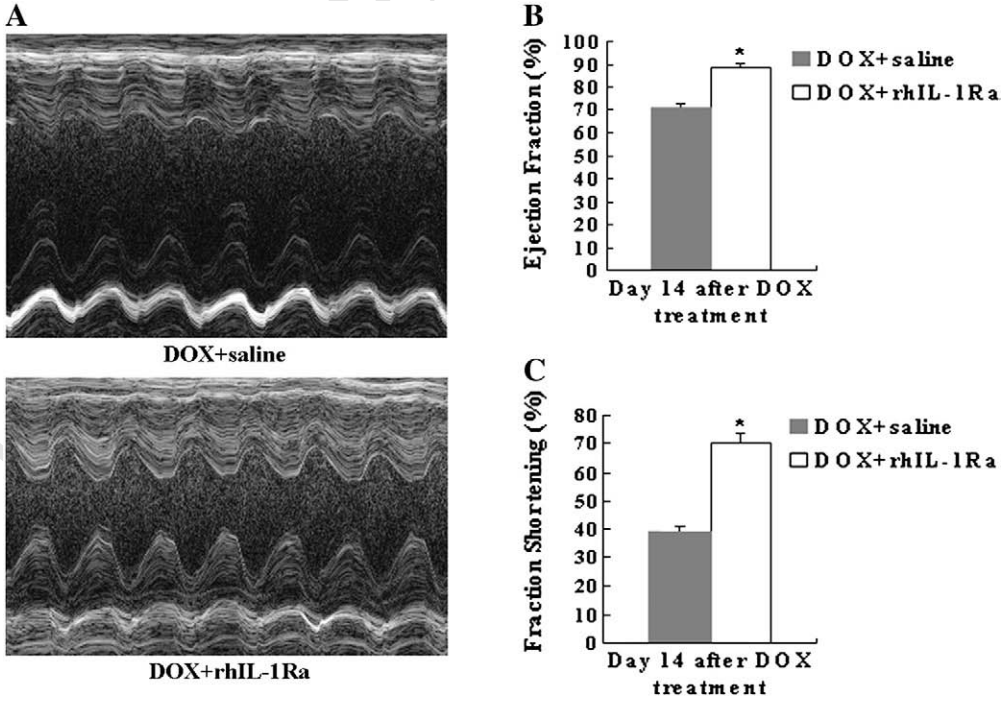

Fig. 4. RhIL-1Ra protects against DOX-induced cardiac function loss. (A) Echocardiographic analysis of cardiac function on day 14 after DOX treatment, images were taken after the mice were anesthetized with 2% isoflurane. Short-axis-motion-mode images were recorded at the papillary muscle level for cardiac functional analysis of the DOX + saline and DOX + rhIL-1Ra groups. (B, C) RhIL-1Ra significantly rescued ejection fraction and fraction shortening after DOX treatment (mean ± S.D. n = 3). *Significantly different compared to the DOX + saline treatment ($P<0.05$).

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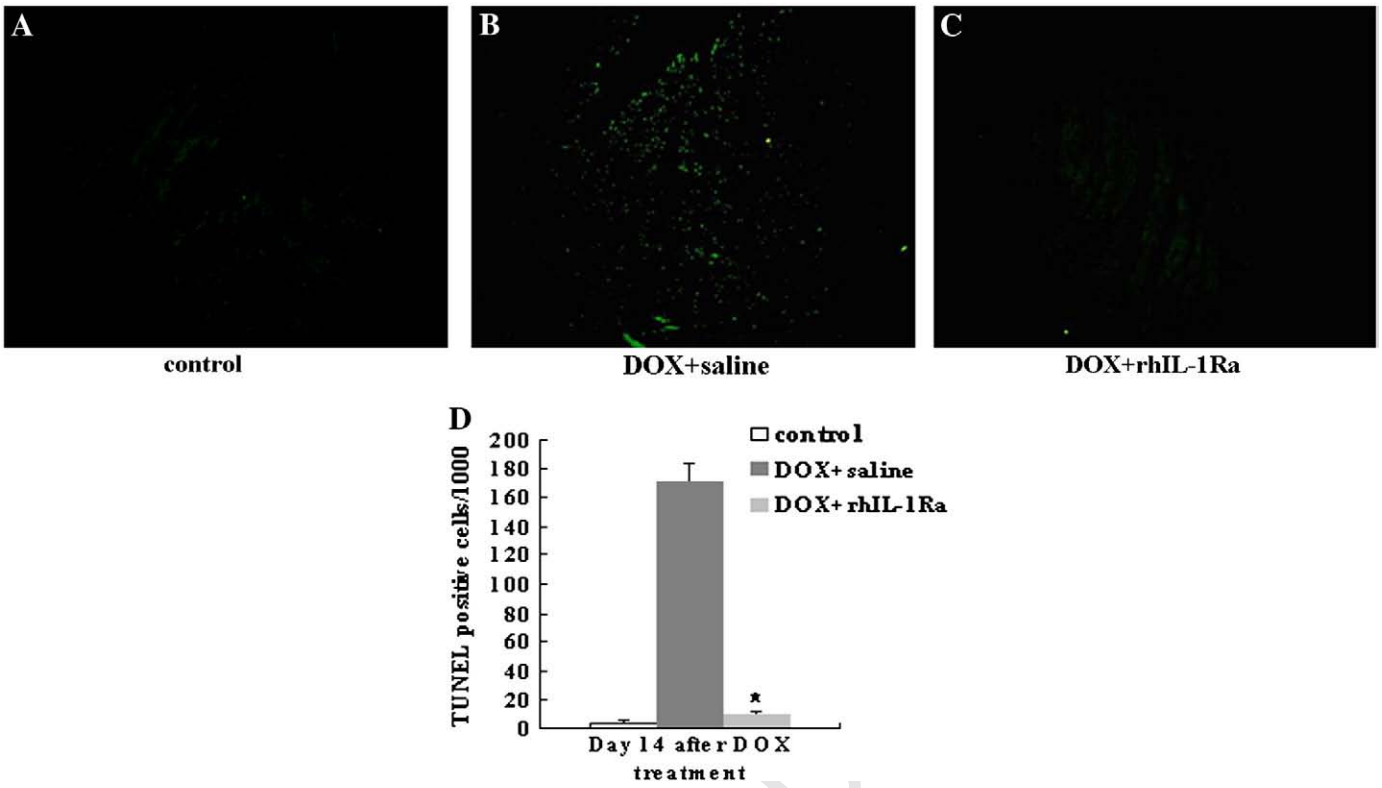

Fig. 5. RhIL-1Ra prevents DOX-induced cardiomyocyte apoptosis. (A, B, C) TUNEL detection of heart tissues from control, DOX + saline and DOX + rhIL-1Ra groups (×200), showing that TUNEL-positive cells were prominent in the DOX + saline group, but less in the DOX + rhIL-1Ra group. (D) Quantitative analysis of apoptotic cell death showed that there were significant decreases of apoptosis cells in the DOX + rhIL-1Ra group after 14 days of DOX treatment, compared to the DOX + saline group (mean ± S.D. n = 3). *Significantly different compared to the DOX + saline group ($P<0.05$).

generate peroxynitrite, a potent oxidant which injures the cell membrane, causes the cell death and activates apoptosis through the injury of mitochondria (Hori and Nishida, 2009). As referred before, the mediating mechanism for the cardiotoxic effect of DOX is the generation of oxidative stress. We assume that IL-1β might increase the damage of DOX-induced cardiotoxicity through the interaction with oxygen free radicals in the heart tissue after DOX treatment.

A series of studies had shown that apoptosis played an important role in DOX-induced cardiotoxicity (Arola et al., 2000). DOX-induced generation of reactive oxygen species had been proved to be responsible for this drug's apoptosis (Kalyanaraman et al., 2002). Determination of apoptosis by the amount of cytosolic mononucleosomal and oligonucleosomal DNA fragments (180 bp or multiples) showed that they were significantly increased after 4 days of one single 20 mg/kg DOX treatment (Childs et al., 2002), indicating that mitochondrial-mediated apoptosis was the underlying mechanism of DOX-induced cardiotoxicity. According to the in-vitro study by Ing et al., after 72 h of IL-1β treatment, IL-1β alone was sufficient to activate neonatal cardiac myocyte apoptosis through the way of stimulating a rapid sustained increase in NO. We suspect that IL-1β might increase DOX-induced cardiac damage via increasing the amount of cardiomyocytes apoptosis. With the method of TUNEL study, we have showed that rhIL-1Ra can reduce DOX-induced cardiomyocytes apoptosis.

One of the limitations in our study is that we don't know how IL-1β excreted after DOX treatment and the exact effect of IL-1β in DOX-induced cardiotoxicity, especially in the first 7 days after DOX treatment. More studies are needed to determine the exact relationship between IL-1β and DOX-induced cardiotoxicity.

In summary, our data show that IL-1 is closely connected with the cardiac damage of DOX treatment and rhIL-1Ra, a natural antagonist of IL-1, provides protection against DOX cardiotoxicity, indicating that combining rhIL-1Ra with DOX could decrease DOX-induced cardiotoxicity in treatment of cancer.

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