Reactive oxygen species regulate FLICE inhibitory protein (FLIP) and susceptibility to Fas-mediated apoptosis in cardiac myocytes

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Abstract

\textbf{Objective:} Fas ligand (FasL) is a key cytokine which initiates apoptosis when FasL binds to its receptor, Fas. Cardiac myocytes are generally resistant to Fas-induced apoptosis. However, sublethal dose of doxorubicin (Dox) can sensitize cardiac myocytes to Fas-induced apoptosis. We investigated the molecular mechanism by which Dox sensitizes cardiac myocytes to Fas-induced apoptosis. FLICE inhibitory protein (FLIP) is a key molecule for blocking Fas-induced apoptosis by functioning as a caspase-8 dominant negative.

\textbf{Methods and results:} FLIP was constitutively expressed in cultured neonatal rat cardiac myocytes. FLIP protein levels were markedly down-regulated by Dox in a time-dependent and dose-dependent manner. Next, we examined the relation of reactive oxygen species (ROS) by Dox to the expression of FLIP. Both of \textit{N}\textendash\textit{acetylcysteine} (NAC) and the combination of superoxide dismutase and catalase restored the decreased FLIP in Dox-treated cardiac myocytes to the basal level. NAC also restored the increased formation of thiobarbituric acid-reactive substance after Dox-treatment. Concurrently, the susceptibility to Fas-mediated apoptosis disappeared with the treatments of the antioxidant agents. Hydrogen peroxide down-regulated FLIP in a dose-dependent fashion and also sensitized cardiac myocytes to Fas-induced apoptosis. 

\textbf{Conclusions:} FLIP, an inhibitor of apoptosis induced by cytokines of TNF family, contributes at least partly to Dox-induced sensitization to Fas-mediated apoptosis in cardiac myocytes. The expression of FLIP in cardiac myocytes is regulated by ROS.

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1. Introduction

Doxorubicin, an anthracycline antibiotic, has been used as a powerful drug in the fight against neoplastic diseases. However, its practical use is sometimes limited by acute and chronic cardiotoxicities [1]. The cardiotoxicity is dose-dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy with fatal congestive heart failure [1]. The exact causal mechanism of doxorubicin-induced cardiomyopathy remains unclear, but most of the evidence indicates that free radicals are involved [2,3]. The chemical structure of doxorubicin is prone to the generation of free radicals, and the oxidative stress correlates with cellular injury [4]. Increased oxidative stress may lead to a variety of subcellular changes in the myocardium.

Apoptosis plays a pivotal role in loss of cells not only during physiological phenomena such as normal turnover of tissues, but also in many pathophysiological phenomena. Evidence is accumulating that the apoptotic mechanism is involved in various heart disorders [5–8]. Fas/Fas ligand system is one of the key regulators of apoptosis [9]. Fas/CD95 is the cell-surface receptor expressed in many cell types including cardiac myocytes [10]. Fas/CD 95 ligand (FasL) is expressed on the membrane of cells, such as activated T cells [11], NK cells [12], endothelial cells

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[13] or immune privileged sites [14]. Both the membrane-bound FasL and its cleaved form, soluble FasL, can induce apoptosis in vitro and in vivo [15–17], while the lethal ability is more potent in the membrane-bound FasL [18]. The Fas/FasL system has been reported to be activated in human heart failure [19–21]. Doxorubicin-induced deterioration in left ventricular performance in rat was inhibited by neutralizing anti-FasL antibody [22], suggesting an important role of Fas/FasL system in doxorubicin cardiomyopathy. However, it is obscure how administration of doxorubicin renders the myocardium prone to Fas-mediated apoptosis. We previously observed that cardiac myocytes were generally resistant to Fas-mediated apoptosis in vitro. However, after treatment of sublethal dose of doxorubicin, cardiac myocyte apoptosis was dramatically facilitated by recombinant FasL [23]. This finding is intriguing, because the doxorubicin-induced sensitivity to Fas in cardiomyocytes can be induced by a molecule which may be a target for treating doxorubicin-associated cardiomyopathy.

Recently, FLICE-inhibitory protein (FLIP), a molecule with sequence homology to caspase-8 (FLICE), was identified as an anti-apoptotic protein [24]. FLIP inhibits Fas- and TNF-mediated apoptosis [24,25]. When FasL binds to Fas, Fas-associated death domain (FADD) is activated and then, caspase-8 is cleaved and activated, leading to subsequent activations of caspase cascades. FLIP is capable of binding FADD, thereby preventing cleavage and activation of caspase-8, thus shutting off the initiation of the death pathway [24–26]. Although high levels of transcripts of FLIP are expressed in the heart compared to other organs [26], little is known about the basic mechanisms controlling the expression of FLIP. In this study, we investigated whether FLIP levels are related to the doxorubicin-induced sensitivity to Fas in cultured neonatal rat cardiac myocytes. Moreover, we examined the hypothesis that the expression of FLIP was down-regulated by oxidative stress.

2. Methods

2.1. Materials

Human soluble recombinant FasL (rFasL) [27] and human Fas chimeric protein (Fas-Fc) [28] were provided by Bioscience Research Laboratory, Mochida Pharmaceutical Co. (Tokyo, Japan). A polyclonal anti-FLIP antibody was generated in a Japanese white rabbit according to the manufactures instructions (Mebstain Apoptosis Kit Direct, MBL, Nagoya, Japan). The cardiac myocytes were fixed, washed, and incubated with distilled water, and then incubated with TdT and FITC-dUTP. Counter staining was performed with propidium iodide (PI) by diluting with PBS to obtain a concentration of 1 μg/ml. Approximately 800–1000 nuclei from random fields were analyzed for each sample. The apoptotic index was calculated as apoptotic nuclei/total nuclei×100 (%). The presence of apoptotic profiles was also confirmed by staining with Hoechst dye #33342.
2.4. Cell viability assay

Cell viability was analyzed by MTT assay [31]. Equal numbers of cardiomyocytes were plated on 96-well plates. MTT (3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide; 0.625 mg/ml) was added to each well. The plates were then analyzed with a multwell plate reader at 590 nm.

2.5. Western blot analysis

Cultured cardiac myocytes were lysed for 30 min on ice in a lysis buffer containing 2% NP-40, 0.5% sodium deoxycholate, 0.2% SDS, and protease inhibitor mixture, and then centrifuged at 12 000 rpm for 10 min at 4 °C. The protein concentration of each sample was measured using BCA protein assay reagents (Pierce Chemical Co., Rockford, IL, USA). BSA was used as a protein assay standard. For SDS–PAGE, 80 μg of protein of each sample was added on 10% polyacrylamide gel and then electrophoretically transferred to PVDF membrane. After blocking with a buffer containing 5% non-fat milk and 0.2% Tween-20 for 1 h at room temperature, the membrane was incubated with either anti-FLIP, anti-Fas, or anti-FADD antibody for 3 h at room temperature. After washing three times with PBS containing 5% non-fat milk, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG or anti-goat IgG for 1 h at room temperature. The membrane was washed again and the signals were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech., Japan).

2.6. Measurement of lipid peroxidation

The extent of lipid peroxidation was determined by using a thiobarbituric acid reactive substance (TBARS) assay in cultured rat neonatal cardiac myocytes. Malondialdehyde (MDA) was used as standard and the results were expressed as nanomol MDA per milligram protein [32].

2.7. Caspase-8 protease assay

Activation of caspase-8 was detected by the caspase-8 colorimetric protease assay kit (MBL, Nagoya, Japan). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNa) after cleavage from the labeled substrate IETD-pNa. IETD-pNa is a specific substrate for activated caspase-8, which has Ile-Glu-Thr-Asp amino acid sequence labeled with p-nitroanilide. The pNa light emission was quantified using a microtiter plate at 405 nm.

2.8. Data and statistical analysis

Data were expressed as mean±S.E. The values were tested by one-way analysis of variance (ANOVA), and was followed by the Scheffe’s F-test. Differences were considered statistically significant at P<0.05.

3. Results

3.1. Doxorubicin-induced sensitization to Fas-mediated apoptosis in neonatal rat cardiac myocytes

The apoptotic index had no significant change from control to treatments of recombinant human FasL (FasL) in neonatal rat cardiac myocytes (Fig. 1A). However, 12 h after pretreatment of sublethal dose of doxorubicin (0.5 μmol/l), FasL markedly increased the apoptotic index in a dose-dependent manner. The FasL-induced apoptosis was completely blocked by anti-Fas antibody and caspase-8 inhibitor (Z-IETD-FMK), indicating that Fas-mediated signaling was activated by FasL after pretreatment of doxorubicin. Similarly, although FasL alone did not affect the viability of cardiac myocytes, FasL reduced the viability of cardiac myocytes in the presence of sublethal dose of doxorubicin (Fig. 1B). Cardiac myocytes were stained with Hoechst dye (Fig. 1C-D). With 1 μg/ml of FasL after treatment of doxorubicin, nuclei of cells showed typical morphology of apoptosis such as nuclear condensation and fragmentation (Fig. 1D).

3.2. Down-regulation of FLIP protein levels by doxorubicin

FasL-induced activation of caspase-8 occurred only after treatment of sublethal dose of doxorubicin in cardiac myocytes [23] (also shown in Fig. 4). Fas protein was constitutively expressed at basal condition and slightly up-regulated by 24 h treatment of doxorubicin (data not shown). FADD protein levels were unchanged by the treatment (data not shown). We assessed expression levels of FLIP, a molecule with sequence homology to caspase-8 and capacity of preventing proteolytic activation of caspase-8. Immunoblotting revealed that untreated cardiac myocytes express a 55-kDa immunoreactive protein that has an identical mobility to the positive control, an extract from COS cells transfected with the FLIP expression plasmid [29]. Doxorubicin (0.5 μmol/l) strongly down-regulated the expression of the FLIP-immunoreactive protein in a time-dependent manner (Fig. 2A). When cardiac myocytes were treated with various concentrations of doxorubicin for 24 h, expression of FLIP protein was down-regulated in a dose-dependent manner (Fig. 2B).

3.3. Antioxidants decrease doxorubicin-induced sensitivity to Fas and recover down-regulation of FLIP levels by doxorubicin

Antioxidants were used to determine whether reactive
oxygen species can contribute to the sensitization to Fas after the treatment of doxorubicin in neonatal rat cardiac myocytes. In the presence of antioxidants, SOD and CAT, the apoptotic index was markedly reduced despite both treatments of doxorubicin and FasL (Fig. 3A). Similarly a scavenger of reactive oxygen species, N-acetylcysteine (NAC), decreased the apoptotic index induced by treatments of doxorubicin and FasL. The SOD and CAT recovered the decreased cell viability by doxorubicin and FasL, and NAC also restored the cell viability (Fig. 3B). The effects of these antioxidants indicate that reactive oxygen species are closely related to the doxorubicin-induced susceptibility to Fas in neonatal rat cardiac myocytes. Next, we examined whether the down-regulated FLIP levels by doxorubicin can be restored by the antioxidants (Fig. 3C). In the presence of NAC and the combination of SOD and CAT, the decreased levels of FLIP by doxorubicin were restored to the untreated levels. As expected with these findings, doxorubicin increased TBARS formation in the cultured cardiac myocytes, and NAC lowered the increased formation of TBARS with doxorubicin (Fig. 3D).

Activity of caspase-8 increased with the addition of FasL after pretreatments of doxorubicin. However, FasL or doxorubicin alone could not activate caspase-8 (Fig. 4). The SOD and CAT abolished the caspase-8 activation induced by FasL and doxorubicin. NAC also abolished the increased caspase-8 activity.

3.4. Hydrogen peroxide down-regulates FLIP levels and increases Fas-mediated apoptosis

To determine whether H$_2$O$_2$ inhibits the expression of FLIP, cardiac myocytes were incubated with exogenously administered H$_2$O$_2$. As shown in Fig. 5, the levels of FLIP were decreased by incubation with H$_2$O$_2$ (50 µmol/l for 12 h) in a dose-dependent manner. Moreover, FasL after pretreatment of H$_2$O$_2$ significantly decreased the cell viability of cardiac myocytes, whereas FasL or H$_2$O$_2$ alone had no significant change in the cell viability of cardiac myocytes (Fig. 5B).

4. Discussion

The mechanisms by which doxorubicin causes cardiotoxicity have been the subject of extensive investigation and debate for more than two decades [1,2]. It has been suggested that apoptosis of cardiac myocytes is involved in the doxorubicin-induced loss of contractile function [22,33,34]. Recently, neutralizing anti-FasL antibody was reported to recover the doxorubicin-induced decrease of left ventricular performance in rat [22] suggesting that doxorubicin-induced apoptosis of cardiac myocytes may be executed through Fas-mediated pathway. Whereas cardiac myocytes were generally resistant to Fas-mediated apoptosis, cardiac myocytes became susceptible to Fas-mediated apoptosis after treatment of sublethal dose of doxorubicin [23]. Caspase-8 activity was markedly increased by FasL only after the pretreatment of doxorubicin indicating that doxorubicin modulates the signal from its receptor Fas to caspase-8. We focused on the behavior of FLIP, an anti-apoptotic molecule by inhibiting activation of caspase-8. Rat neonatal cardiac myocytes constitutively expressed FLIP protein, which is consistent with the resistance of cardiac myocytes to Fas-mediated apoptosis despite Fas expression in the untreated cardiac myocytes [10]. However, treatment of doxorubicin down-regulated the protein level of FLIP in neonatal rat cardiac myocytes in a time- and dose-dependent manner. Doxorubicin-induced downregulation of FLIP is one possible mechanism by which cardiomyocytes are prone to Fas-mediated apoptosis.

Doxorubicin has been reported to produce reactive oxygen intermediates including hydroxyl radicals and superoxide as well as hydrogen peroxide [4]. Doxorubicin also decreased protein levels of intracellular antioxidant enzymes, glutathione peroxidase (GSH) and manganese SOD [35]. The myocardial redox balance is markedly impaired by doxorubicin. Reactive oxygen species produced by doxorubicin play a role in cell death of cardiac myocytes [34]. Lower concentrations of doxorubicin produced apoptotic cell death of cultured cardiac myocytes [33,34,36] and there appears to be a switch from apoptosis to necrosis at doxorubicin concentrations of 2–10 µmol/l [33,34]. In addition to doxorubicin concentration, cultured conditions, species and aging are important factors of cell survival of cardiac myocytes. In this study, 0.5 µmol/l of doxorubicin did not induce an apparent decrease in cell viability of neonatal rat cardiac myocytes. Nevertheless, 0.5 µmol/l doxorubicin down-regulated FLIP expression to a critical level at which cardiac myocytes became susceptible to FasL-induced apoptosis.

Although valuable information regarding signal transduction pathways leading to apoptosis has been obtained from the study using neonatal cardiac myocytes, there is
little information regarding the mechanisms for apoptosis wherein this process occurs in the adult heart. Results from the in vitro study using neonatal cardiomyocytes can not always be applied to the adult intact heart.

One of the most important findings in the present study is the profound inhibition of FLIP expression by reactive oxygen species. Combination of superoxide dismutase (SOD) and catalase, which are antioxidant enzymes hydro-
Fig. 3. Antioxidants abolished apoptosis of cardiac myocytes induced by recombinant Fas ligand (FasL) after treatment of doxorubicin (Dox; 0.5 μmol/l).

(A) Cardiomyocyte apoptosis was estimated by TUNEL staining in the presence of N-acetylcysteine (NAC) and the combination of superoxide dismutase (SOD) and catalase (CAT). Apoptotic index was calculated as a ratio of apoptotic cells to total cell number. (B) Cell viability was determined by MTT assay in the presence of NAC and the combination of SOD and CAT. Results are expressed as mean±S.E.M. of three separate experiments. (C) Antioxidants restored the expression of FLIP (55 kDa) in cardiac myocytes treated with Dox (0.5 μmol/l). FLIP levels were determined by Western blot analysis in cardiomyocytes incubated with doxorubicin in the presence of NAC and the combination of SOD and CAT. FLIP expression was quantified by densitometry and presented as mean±S.E.M. from three independent experiments. (D) Oxidative stress by Dox was assessed by TBARS assay. NAC abolished the increase of MDA formation by Dox. Concentrations were as follows: SOD 500 U/ml, CAT 500 U/ml, and NAC 50 μmol/l.
Fig. 4. Enzymatic activities of caspase-8 in cardiac myocytes. Caspase 8 activity was dramatically enhanced after both treatments of recombinant Fas ligand (FasL: 1 μg/ml) and doxorubicin (Dox: 0.5 μM). N-acetylcysteine (NAC) and the combination of superoxide dismutase (SOD) and catalase (CAT) abolished the enhancement of caspase-8 activity after both treatments of FasL and doxorubicin. Concentrations were as follows: SOD 500 U/ml, CAT 500 U/ml, and NAC 50 μmol/l. Results are expressed as mean±S.E.M. of three separate experiments.

Fig. 5. Hydrogen peroxide (H₂O₂) down-regulated the expression of FLIP and sensitized cardiac myocytes to Fas. (A) The expression of FLIP, (55 kDa) was determined by Western blot analysis in cardiomyocytes incubated with various doses of hydrogen peroxide for 24 h. FLIP expression was quantified by densitometry. (B) Cardiac myocytes were incubated with human recombinant Fas ligand (FasL: 1 μg/ml) for 12 h after 12 h treatment with H₂O₂ (50 μmol/l). Cell viability was determined by MTT assay. *P<0.01 vs. untreated cells. Results are expressed as mean±S.E.M. of three separate experiments.

lyzing superoxide and hydrogen peroxide, respectively, prevented the doxorubicin-induced decrease of FLIP protein expression. Likewise, the antioxidant N-acetylcysteine (NAC) also restored the down-regulation of FLIP with doxorubicin treatment. Conversely, hydrogen peroxide, which is the most diffusible reactive oxygen species, itself decreases FLIP expression in a dose dependent manner. These results indicate that reactive oxygen species mediate the inhibitory action of doxorubicin on FLIP expression in cardiac myocytes. Following the reversal to the baseline level of FLIP by treatments with antioxidants, cardiac myocytes became resistant to Fas-mediated apoptosis again. Levels of FLIP have been demonstrated to play a pivotal role in the Fas-mediated apoptosis in various cell types [24–26,37]. Thus, reactive oxygen species produced by doxorubicin sensitize rat neonatal cardiac myocytes to Fas-mediated apoptosis at least partly by the way of down-regulating FLIP. In human Jurkat T cell lines, doxorubicin-induced apoptosis was reported to be independent of Fas signaling [38,39]. However in leukaemic cells [40], incubation with anti-Fas IgM antibody and doxorubicin augmented apoptotic responses as we demonstrated using neonatal rat cardiomyocytes in the present study. These data implicate that doxorubicin- and Fas-mediated apoptosis is cell specific events and may share some common components in signaling pathways leading to apoptosis. To our knowledge, precise mechanisms for the downregulation of FLIP by reactive oxygen species are still unknown. Transcriptional regulations and the protein stability or degradation of FLIP are suggested as possible mechanisms [41,42].

Besides doxorubicin-induced heart failure, reactive oxygen species have been shown to cause contractile failure and structural damage in various types of heart failure [43]. There may be a possibility that FLIP levels are downregulated by reactive oxygen species in the myocardium of the failing heart. Recently, Imanishi et al. [44] observed that FLIP-positive cells rarely had fragmented DNA, while TUNEL-positive cells rarely contained FLIP in end-stage human hearts. Cardiac myocytes of the failing heart may be primed for apoptotic signal by FasL which has been reported to be elevated in patients with congestive heart failure [21] (Fig. 6). In the transgenic mouse overexpressing FasL in the heart, neither myocardial apoptosis nor necrosis occurred in the heart despite expression of Fas [45]. However, since the FLIP level should be unchanged in the transgenic mouse, the absence of apoptosis in the
FasL-enforced myocardium might be due to relatively high FLIP levels.

In conclusion, FLIP contributes, at least in part, to doxorubicin-induced susceptibility to Fas-mediated apoptosis in cardiac myocytes. Importantly, the expression of FLIP is regulated by reactive oxygen species in cardiac myocytes.

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References


