Fas-Mediated Apoptosis in Adriamycin-Induced Cardiomyopathy in Rats
In Vivo Study

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Background—The precise molecular mechanism of Adriamycin-induced cardiomyopathy (ADR-CM) is still unknown. We address the demonstration of apoptotic myocardial cell death and the apoptosis-inducing molecules in ADR-CM induced in rats.

Methods and Results—Until 8 weeks after the first administration of ADR, there was no increase in the number of labeled cells by terminal deoxynucleotidyl transferase assay (TUNEL method). Apoptotic indices increased significantly at weeks 9 and 10 in hearts of the ADR-treated group but not in those of the control group (0.42 ± 0.12% versus 0.10 ± 0.02% and 0.86 ± 0.11% versus 0.09 ± 0.04% at weeks 9 and 10, respectively). DNA ladder formation was also observed in the myocardial tissues during the late stages of the ADR-CM of rats. There was no significant difference in expression of \( p53 \) gene between the ADR group and the control group at either the message or the protein level. An overexpression of Fas antigen was shown in myocardial cells of ADR-treated hearts at weeks 9 and 10 by both Western blotting and immunofluorescent staining. Furthermore, we confirmed that neutralization of anti–Fas ligand antibody inhibited ADR-induced apoptosis.

Conclusions—Apoptotic cell death was observed in the hearts of ADR-CM rats, and the number of apoptotic myocardial cells increased with the deterioration of morphological findings and cardiac function, indicating that apoptosis may be an important mechanism of loss of myocardial cells and cardiac dysfunction in ADR-CM. Apoptosis in ADR-CM rats is not p53-dependent but rather is executed through a Fas-mediated pathway. (Circulation. 2000;102:572-578.)

Key Words: apoptosis • cardiomyopathy • doxorubicin hydrochloride

Doxorubicin HCl (Adriamycin, ADR) is one of the most effective and useful antineoplastic agents for the treatment of a variety of malignancies, including lymphoma, leukemia, and solid tumors. However, its practical therapeutic use is sometimes limited by late-onset and acute and chronic cardiotoxities. The chronic cardiotoxicity is dose-dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy (DCM) with fatal congestive heart failure. Previous studies on the pathogenesis of ADR-induced cardiomyopathy (ADR-CM) have reported that free-radical–mediated myocyte damage, intracellular calcium overload, disturbance in myocardial adrenergic function, and release of cardiotoxic cytokines might be involved in the pathogenesis of ADR-CM. ADR causes the selective inhibition of gene expression specific for cardiac muscles, such as \( \alpha \)-actin, troponin, myosin light chain-2, and the M isoform of creatine kinase, that may explain the myofibrillar loss associated with ADR-CM.

Apoptosis plays a pivotal role in loss of cells not only during physiological phenomena, such as embryogenesis and normal turnover, but also in many pathological processes. Evidence is accumulating that the apoptotic mechanism is involved in loss of myocytes in various human heart disorders, including DCM, chronic myocarditis, and myocardial infarction. As to apoptosis-related genes, \( bax, bcl-2, \) and Fas antigen were shown to be overexpressed in myocytes of DCM, chronic heart failure, and myocardial infarction, although the precise mechanism of apoptosis induction in those heart disorders is still to be investigated. In ADR-CM, however, the significance of apoptotic cell death and the apoptosis-related genes involved have not yet been investigated.

The purpose of our present study was to investigate whether apoptotic cell death contributes to the loss of myocytes in ADR-CM and to analyze the mechanism of apoptosis induction in ADR-CM, concentrating on p53 and Fas antigen, which are 2 important apoptosis-inducing molecules, in an ADR-CM model induced in rats.
Methods

Experimental Animals
ADR-CM was induced in male Wistar rats weighing 276.6±5.7 g by weekly administration of 2 mg/kg of ADR (supplied from Kyowa Hakko Kogyo Co Ltd) via a tail vein for 8 weeks according to Podesta et al.16 Instead of ADR, the same volume of physiological saline was injected into control rats. Batches of 3 rats were euthanized 24 hours after the beginning of administration of ADR or saline and also on the same day of weeks 5, 8, 9, and 10. Week 0 is taken to be the time of the first administration of ADR. After anesthesia with ether, the chest was opened and the heart immediately isolated. The blood in the heart was removed and the heart weighed. Ventricles were cut horizontally and separated into 3 slices. The midslice of the ventricle was fixed in 4% paraformaldehyde at 4°C for 8 hours, dehydrated in graded alcohol series, and then embedded in paraffin. The apical and basal portions of the left ventricle (LV) were rapidly frozen in liquid nitrogen and were preserved at −80°C until use. A part of the LV was also embedded in OCT compound and rapidly frozen.

Histological Assessment of Myocardial Damage
Paraffin sections 3 μm thick were stained with hematoxylin and eosin, reticulin silver impregnation, and Azan-Mallory stain.

Analysis of LV Performance
LV performance was examined by echocardiography at weeks 9 and 10 after the first injection of ADR or saline. LV dimensions (end-diastolic diameter [LVDd] and end-systolic diameter [LVDs]) were measured by echocardiogram with an HP SONOS 1000 with a 7.5-MHz transducer (Hewlett-Packard Co). The percent fractional shortening (%FS) of LV was calculated from the formula %FS=[(LVDd−LVDs)/LVDd]×100.

In Situ Terminal Deoxynucleotidyl Transferase Assay (TUNEL Method)
The TUNEL method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-OH ends of DNA and the ensuing synthesis of a polydeoxynucleotide polymer. The TUNEL method was applied to 4% paraformaldehyde–fixed, paraffin-embedded sections 3 μm thick with an ApoTag In Apoptosis Detection Kit (Oncor) according to the manufacturer’s instructions.

Quantitative Analysis of DNA Strand Breaks in Myocytes
Myocardial nuclei of 3-μm horizontal sections of the heart, including the left and right ventricles and intraventricular septum, were labeled by the TUNEL method. Their numbers were counted, and an apoptotic index (number of myocardial nuclei labeled by the TUNEL method/number of total myocardial nuclei) was calculated. The numbers of apoptotic cells were counted by 3 persons, 1 pathologist and 2 pediatricians, without knowledge of other information about the samples.

DNA Gel Electrophoresis
DNA was extracted from fresh-frozen myocardial tissues. Tissues were homogenized and lysed in a solution containing 50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L EDTA, 100 mmol/L NaCl, and 1% SDS and digested in 0.5 mg/mL protease K (Sigma Chemical Co) at 55°C for 16 hours. RNAse A (Sigma Chemical Co) was added to each sample to achieve a final concentration of 0.2 mg/mL of RNAse A, and the samples were incubated at 37°C for 2 hours. After phenol/chloroform extraction, DNA was precipitated by ethanol and dissolved in TE solution (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA). DNA samples (10 μg) were subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination.

Reverse Transcription–Polymerase Chain Reaction Analysis
Total RNA was extracted from fresh-frozen myocardium by the Isogen RNA extraction system (Nippon Gene). cDNA was synthesized from 5 μg of each RNA sample by use of random hexamers and reverse transcriptase at 37°C for 30 minutes. Rat p53 mRNA was amplified with a Multiplex polymerase chain reaction (PCR) kit (Maxim Biotech Inc) and rat G3PDH with the following primers: 5’-TGAAGGTCGGAGTCAACGGATTGGT-3’, 5’-CATGTGG-GCCATGAGTGCCACCC-3’ (983-bp products). Cycling parameters were as follows: the cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 60°C, and 1 minute of extension at 74°C. PCR amplification for 30 cycles, which was determined to be within the linear range of product amplification for both p53 and G3PDH mRNA, was carried out for semiquantitative PCR analysis. Then, 2.5 μL of the PCR products was analyzed by electrophoresis on a 1% agarose gel, followed by ethidium bromide staining to determine the levels of p53 mRNAs relative to the control G3PDH transcript. Further verification of the PCR product identity was determined by comparison with standards supplied by the kit manufacturer (Maxim Biotech Inc).

Western Blot Analysis
Proteins were extracted from fresh-frozen LV myocardium. Homogenized myocardial tissue was lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.2), 50 mmol/L NaCl, 1% Nonidet P-40, 1% sodium deoxycholic acid, and 2% SDS with protease inhibitor cocktail (Boehringer-Mannheim). Protein concentration was evaluated against standard BSA with a Bio-Rad assay reagent.17 Twenty micrograms of each protein preparation was electrophoretically separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then incubated with either anti-p53 protein (Nichirei, diluted at 1:2000), anti-Fas antigen antibody (Transduction Laboratories, diluted at 1:2500), or anti–β-actin antibody (Sigma Chemical Co, diluted at 1:2000) for 1 hour at room temperature. Western blots were exposed on an x-ray film with an enhanced chemiluminescence kit (Pierce). Bands were identified by comparison to standards purchased from the same companies that supplied antibodies.

Immunofluorescence Stain for Fas Antigen
Frozen sections 4 μm thick were briefly fixed in 4% paraformaldehyde, and anti-Fas antibody (Transduction Laboratories) was applied for 1 hour at room temperature after blocking with 10% normal goat serum. Slides were incubated in FITC-conjugated goat anti-mouse IgG diluted at a ratio of 1:50 for 30 minutes and observed with an Olympus BX60-FLB.

Inhibition of ADR-Induced Apoptosis by Neutralizing Anti–Fas Ligand Antibody
Initially, we investigated by Western blot whether anti–mouse Fas ligand (Fas L) (Pharmingen) cross-reacted to rat. Anti–Fas L antibody, a similar amount of normal mouse IgG serum was injected into control rats. Batches of 4 rats underwent echocardiography and were euthanized 6 hours after the last injection of anti–Fas L antibody. Sections of the heart 3 μm thick were fixed in 4% paraformaldehyde and embedded in paraffin as stated above. Myocardial nuclei were labeled by the TUNEL method, and an apoptotic index was calculated.

Data Collection and Analysis
All results are presented as mean±SD. Statistical significances for comparisons of cardiac weights, %FS, and apoptotic ratios between the ADR-injected group and the control group were determined with Student’s t test. Differences in apoptotic ratios among ADR-injected groups were statistically analyzed with Scheffé’s method. Values of P<0.05 were considered to be significant.
Results

Both body and cardiac weights of ADR-injected rats were significantly decreased at week 8 after the first administration of ADR compared with those of the control group. Pleural effusions began to appear in 2 of 3 ADR-injected rats at week 9 and were observed in all rats at week 10 after the first administration (Table 1).

LV Performance

The %FS of the LV diminished significantly in ADR-injected rats at weeks 9 and 10 compared with those of control rats (28.5±4.3% versus 41.5±1.3% at week 9 and 23.1±5.0% versus 39.2±0.4% at week 10, respectively, P<0.005 at each week) (Table 3).

Histopathology

No histopathological difference between the ADR-injected group and the control group was detected until 8 weeks after the beginning of administration of ADR or saline, when vacuolation and myofibrillar loss appeared to be observed in focal areas of ADR-injected rats. In the ADR-injected group, the histological degenerative changes rapidly increased at weeks 9 and 10, in association with compensatory myocardial hypertrophy and interstitial fibrosis (Figure 1A). Pyknotic nuclei and fragmentation of the nuclei were sometimes found in the ADR-injected group (Figure 1A and inset).

Apoptotic Cells

The TUNEL method labeled not only fragmented nuclei of myocardial cells but also pyknotic ones. A small number of cells labeled by the TUNEL method were detected even in the control group and in the early stages of ADR-treated hearts. The number of labeled nuclei increased significantly in ADR-injected rats during the late stages, especially at weeks 9 and 10, compared with those of controls (Figure 2A and 2B; Table 2). Apoptotic ratios of ADR-injected versus control rats were 0.42±0.12% versus 0.10±0.02% at week 9 and 0.86±0.11% versus 0.09±0.04% at week 10, respectively, and a statistically significant increase in the ratio of labeled cells was detected at week 10 after the beginning of ADR administration (P<0.05).

DNA Gel Electrophoresis

DNA laddering with the lowest band at 180 bp, indicating nucleosomal breakdown of genomic DNA, was seen in the ADR-injected group at week 10, whereas no DNA fragments were detected in the control group at any stage (Figure 3).

Expression of p53 Gene

mRNA of the p53 gene was detected in both the control and the ADR-injected groups by reverse transcription (RT)-PCR. A semiquantitative study disclosed no difference in expression of p53-mRNA between the 2 groups (Figure 4A). Furthermore, there was no difference in expression of the p53

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**TABLE 1. Body and Cardiac Weights and Occurrences of Pleural Effusion in ADR-injected and Control Rats**

<table>
<thead>
<tr>
<th></th>
<th>ADR-Treated Rats</th>
<th>Control Rats</th>
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<tr>
<td></td>
<td>Body Weight, g</td>
<td>Cardiac Weight, g</td>
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<tr>
<td>24 h</td>
<td>276.6±5.7</td>
<td>ND</td>
</tr>
<tr>
<td>Wk 5</td>
<td>311.6±6.4*</td>
<td>0.65±0.08†</td>
</tr>
<tr>
<td>Wk 8</td>
<td>258.3±25.5†</td>
<td>0.73±0.17†</td>
</tr>
<tr>
<td>Wk 9</td>
<td>256.3±24.9†</td>
<td>0.76±0.08†</td>
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<tr>
<td>Wk 10</td>
<td>246.6±18.8†</td>
<td>0.50±0.09†</td>
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ND indicates not determined. Data are mean±SD of 3 rats.

*Significantly different from control (P<0.01).
†Significantly different from control (P<0.001).

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**Figure 1.** Light microscopic findings of LV of ADR-treated myocardium at week 10 after beginning of administration of ADR or saline. Vacuolization and hypertrophy are seen in ADR-injected rat heart (A). Pyknotic and fragmented myocardial nucleus is observed in inset. Hematoxylin-eosin stain; magnification: A and B, ×50; inset, ×160.
gene at the message level during the experimental course of the ADR-treated group. Western blot analysis showed no difference in the expression of the p53 protein between the ADR-injected and control groups (Figure 5B).

Expression of Fas Antigen

Until 5 weeks after the first administration, no difference was detected by Western blot in the expression of Fas antigen at the protein level between the control and the ADR-injected groups. However, at weeks 9 and 10, Fas antigen was overexpressed in the ADR-injected hearts compared with the control hearts (Figure 5A). In the ADR-injected rats, fluorescent staining showed dominant overexpression of Fas antigen on the cell membrane of some myocardial cells in the late stages (Figure 6A and 6B).

Inhibition of Apoptosis by Neutralizing Anti–Fas L Antigen

The neutralizing anti–mouse Fas L antibody recognized rat Fas L by Western blot (data not shown). The %FS of the LV in rats treated with anti–Fas L antibody plus ADR versus rats treated with ADR only was 45.3±5.0% versus 32.6±3.4%, and statistically significant improvement of %FS was detected in rats treated with anti–Fas L antibody (P<0.01) (Table 3). Furthermore, apoptotic indexes (number of apoptotic cells) of rats treated with anti–Fas L antibody plus ADR versus rats treated with ADR only were 0.25±0.01% (44.7±4.1%) versus 0.37±0.06% (57.7±9.9%), and a statistically significant decrease of the ratio in labeled cells was detected in rats treated with anti–Fas L antibody plus ADR (P<0.05).

Discussion

This study indicates that apoptosis may play an important role in ADR-CM. In the present study, we used a model of ADR-CM–induced rats, because it has been reported that

<table>
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<th>TABLE 2. Number of Apoptotic Cells in the ADR-Treated Group and Control Group During the Course of the Experiment</th>
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<td>ADR-Treated Myocardia</td>
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<tr>
<td>Number of apoptotic cells</td>
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<tr>
<td>Apoptotic index, %</td>
</tr>
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</table>

Apoptotic index, %, indicates number of myocardial nuclei labeled by the TUNEL method/number of total myocardial nuclei. Data are mean±SD of 3 rats.

*Significantly different from control (P<0.05).
ADR-CM could be established reproducibly in rats. The present ADR-CM model in rats showed histological changes similar to those in human ADR-CM: degeneration and loss of myocytes, compensatory hypertrophy of residual myocytes and interstitial fibrosis. These histological findings deteriorated according to the cumulative dosages of ADR equivalent to the human ADR-CM dose. Diminished LV function demonstrated by echocardiogram and decreased cardiac weights with the appearance of pleural effusion in the late stages of the ADR-treated heart further support the usefulness of the present rat ADR-CM as an experimental model for the investigation of the mechanism of ADR-CM occurring in humans.

With the ADR-injected group, fragmented nuclei, in addition to pyknotic ones, were sometimes observed in myocar-
dial cells in the late stages, suggesting the possibility of apoptotic death. Several recent reports have described myocardial cell death through apoptotic mechanisms in a variety of cardiac diseases and pathophysiological states: end-stage heart failure due to DCM and ischemic cardiomyopathy, myocardial infarction, reperfusion injury in rabbits; myocardial infarction in rats; and hypoxia in rats. In the present study, apoptotic death of myocardial cells was demonstrated in ADR-CM–induced rats by both the TUNEL method and DNA agarose-gel electrophoresis. With accumulation of ADR, the increase in the number of apoptotic myocardial cells seems to be concomi-
tant with the progression of ADR-CM both morphologically and functionally, indicating that apoptotic myocardial cell death may also play an important role in the pathogenesis of ADR-CM in rats.

Our findings differ from those of a previous report regarding the proportion of apoptotic cells in human end-stage heart failure described by Narula et al. The percentages of apoptotic cells in the present study (0.86 ± 0.11% at week 10) are much smaller than those reported by Narula et al (5% to 35.5%). If we take into consideration that apoptosis is completed in at most a few hours, the high values of the apoptotic index reported by Narula et al would be incompatible with real life and would be unreliable, as pointed out by Olivetti et al. Our findings appear to be consistent with the apoptotic ratio of 0.23 ± 0.20% in the failing human heart reported by Olivetti et al. Because most rats in this study died within 12 weeks of the first administration of ADR due to heart failure, our values of the apoptotic index would be the upper limit during the late stage of congestive heart failure.

In the present study, apoptosis was counted as 0.1% (0.09% to 0.14%) even in the hearts of the control group. Fragmented nuclei of cardiomyocytes on the TUNEL stains were distinguished from infiltrating lymphocytes by immunohistochemistry of lymphocyte common antigen (data not shown).

Apoptosis-induction mechanisms were recently separated into p53-dependent and p53-independent pathways. p53 is a cell cycle–regulatory molecule. Many exogenous stimuli, including genotoxic agents, promote the accumulation of the p53 protein in the nucleus, which induces growth arrest and apoptosis. This study examined whether apoptosis induced by ADR in rats was dependent on overexpression of p53. There were no significant differences of the expression of p53 between the ADR and the control groups at either the message or the protein level, suggesting that apoptotic cell death of ADR-CM induced in rats is p53-independent. In
previous reports regarding apoptosis of cardiomyocytes, overexpression of Fas antigen, bcl-2, and bax have been demonstrated in myocardial tissue with infarction, \textsuperscript{14,15} DCM, \textsuperscript{11} hypoxia, \textsuperscript{23} and chronic heart failure. Bax, a conserved homolog of bcl-2, heteromerizes in vivo with bcl-2, which accelerates apoptosis. \textsuperscript{27} Miyashita and Reed \textsuperscript{28} recently demonstrated that p53 is a transcriptional activator of the human bax gene. We have also found that bax is overexpressed in cardiomyocytes of ADR-CM induced in rats (data not shown). According to the present finding on p53, it appears that a novel p53-independent pathway is working in activating transcription of the bax gene in ADR-CM–induced rats.

Fas antigen was demonstrated by both immunofluorescent staining and Western blotting to be overexpressed in the ADR-CM rat model. It has been reported that Fas antigen belongs to the tumor necrosis factor receptor superfamily \textsuperscript{29} and that apoptosis could be induced by ligation of Fas by Fas L. \textsuperscript{29–32} Furthermore, overexpression of Fas antigen has been reported in a variety of conditions: cardiomyopathy induced by rapid pacing in dogs, myocardial infarction in rats, \textsuperscript{14} or hypoxia in cultured neonatal rat cardiomyocytes. \textsuperscript{23} However, no reports have documented a cause-and-effect relationship between apoptosis and Fas antigen in heart diseases. Recently, some investigators reported that induction of Fas L and upregulation of Fas after treatment with anticancer drugs, including ADR, had been observed in a variety of tumor cell lines and that blockade of the Fas/Fas L interaction by an antagonistic antibody inhibited drug-induced cell death in vitro. \textsuperscript{33–36} Kamitani et al \textsuperscript{37} reported that high-molecular-mass Fas aggregates (\textgtrsim200 kDa) were immunoprecipitated with antibodies against the extracellular domain and detected in Western blotting under reducing conditions with antibodies against the death domain or the carboxyl terminus. However, we could detect only small Fas aggregates, not large ones (\textgtrsim200 kDa), because only anti–extracellular domain antibodies were used. In this study, overexpression of Fas antigen was associated with increased numbers of apoptotic myocytes, and neutralizing anti–Fas L antibody not only induced the prevention of deterioration of LV performance but also decreased the number of apoptotic myocytes. These results indicated that ADR-induced myocyte death occurred through a Fas-dependent pathway in ADR-CM. Thus, ADR not only kills the tumor cells acutely but also facilitates myocardial cell death at the late stage via a Fas-mediated pathway. Fulda et al \textsuperscript{33} reported that mitochondria played a central role in the regulation of drug-induced apoptosis by controlling activation of the caspase cascade. However, it has been reported that one of the pathogeneses of ADR-CM might be a histochemochemical mitochondrial effect. \textsuperscript{5} Mitochondrial function might also play a central role in the regulation of ADR-induced myocardial cell death in ADR-CM. Our findings support a possibility of preventing ADR-CM clinically by blockade of the Fas/Fas L interaction by neutralizing either Fas L antibody or soluble Fas.

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References
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