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Anthracycline-Induced Suppression of GATA-4 Transcription Factor: Implication in the Regulation of Cardiac Myocyte Apoptosis

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ABSTRACT

Anthracyclines are effective cancer chemotherapeutic agents but can induce serious cardiotoxicity. Understanding the mechanism of cardiac damage by these agents will help in development of better therapeutic strategies against cancer. The GATA-4 transcription factor is an important regulator of cardiac muscle cells. The present study demonstrates that anthracyclines can down-regulate GATA-4 activity. Treatment of HL-1 cardiac muscle cells or isolated adult rat ventricular myocytes with anthracyclines such as daunorubicin and doxorubicin decreased the level of GATA-4 DNA-binding activity. The mechanism of decreased GATA-4 activity acts at the level of the GATA-4 gene, because anthracyclines caused significantly decreased levels of GATA-4 protein and mRNA. The rate of decline in GATA-4 transcript levels in the presence of actinomycin D was unaltered by anthracyclines, indicating that these agents may affect directly GATA-4 gene transcription. To determine whether decreased GATA-4 levels are functionally related to cardiac muscle cell death that can be induced by anthracyclines, the ability of ectopic GATA factors to rescue anthracycline-induced apoptosis was tested. Adenovirus-mediated expression of either GATA-4 or GATA-6 was sufficient to attenuate the incidence of apoptosis. Furthermore, suppression of GATA-4 DNA-binding activity by a dominant negative mutant of GATA-4 induced the apoptosis. These results suggest that the mechanism of anthracycline-induced cardiotoxicity may involve the down-regulation of GATA-4 and the induction of apoptosis.

The anthracycline antibiotics, including daunorubicin (DNR) and doxorubicin (DOX), have been used for cancer chemotherapy for more than 20 years. DNR is the first anthracycline developed and has been found to be effective against acute leukemia, whereas DOX was found to be effective also against solid tumors. Despite the usefulness of these agents in eliminating cancer cells, severe cardiotoxicity occurs in >20% of patients treated with anthracyclines (Singal and Iliskovic, 1998), causing a significant clinical problem. Cardiac events may include mild blood pressure changes, thrombosis, electrocardiographic changes, arrhythmia, myocarditis, pericarditis, myocardial infarction, cardiomyopathy, cardiac failure, and congestive heart failure. These may occur during or shortly after treatment, within days or weeks after treatment, or may not be apparent until months or even years after completion of chemotherapy. Attenuating anthracycline actions on the heart is expected to have a tremendous impact on the treatment of cancer.

The mechanism by which anthracyclines cause irreversible myocardial damage remains unclear but may involve the generation of reactive oxygen species (ROS), lipid peroxidation, mitochondrial impairment, and modulation of gene transcription. Recent data demonstrated that anthracyclines induce apoptosis of cardiac myocytes (Arola et al., 2000; Kang et al., 2000).

GATA-4 is a member of the GATA family of zinc finger transcription factors, which plays important roles in transducing nuclear events that modulate cell lineage differentiation during development. Six GATA family members have been identified and shown to alter transcription of target genes via binding to the consensus 5′-WGATAR-3′ sequence. Three members of this family, GATA-4, -5, and -6, are ex-

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ABBREVIATIONS: DNR, daunorubicin; DOX, doxorubicin; ROS, reactive oxygen species; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; PCR, polymerase chain reaction; PARP, poly(ADP-ribose) polymerase; TNFα, tumor necrosis factor-α.

pressed in the heart. Functionally relevant GATA-binding sites have been identified in numerous cardiac transcriptional regulatory regions (Charron and Nemer, 1999; Molkentin, 2000). Anthracyclines have been shown to suppress expression of several GATA-regulated genes, including cardiac adriamycin-responsive protein (Jeyaseelan et al., 1997), brain, and atrial natriuretic proteins (Chen et al., 1999), α-myosin heavy chain (Saadane et al., 1999) and calsequestrin (Arai et al., 1998). However, the effects of anthracyclines on GATA transcription factors have not been studied.

In addition to regulating differentiation, there is increasing evidence that GATA factors also control cell survival and apoptosis. Weiss and Orkin (1995) reported that the GATA-1-deficient erythroid precursors undergo apoptosis. In erythroleukemia cells, the induction of apoptosis by estrogen was dependent on the inhibition of GATA-1 (Blobel et al., 1995; Blobel and Orkin, 1996). GATA-1 has been shown to regulate the expression of antiapoptotic proteins Bcl-2 (Tanaka et al., 2000) and Bcl-x_L (Grillot et al., 1997; Gregory et al., 1999). GATA elements were also found in promoters of nitric-oxide synthases (Zhang et al., 1995; Keinanen et al., 1999) and antioxidant enzymes (O'Prey et al., 1993), which may be involved in antiapoptotic activities. GATA-2 is also required for growth and survival of early hematopoietic cells (Tsai et al., 1994; Tsai and Orkin, 1997). In vitamin A-deficient avian embryos, GATA-2 fails to be expressed normally and this is associated with activation of apoptosis (Ghatpande et al., 2002). GATA-6 is involved in controlling apoptosis of colorectal cancer cells by its inhibition of the 15-lipoxygenase-1 gene (Shureiqi et al., 2002). GATA-4 may play a role in regulating apoptosis and cell survival, because the apoptosis of ovarian cells was found to be associated with a decrease in the expression of GATA-4 (Heikinheimo et al., 1997). Furthermore, a lack of GATA-4 is associated with activation of apoptosis in the presumptive foregut (Ghatpande et al., 2000). Endothelin-1, a survival factor for cardiac myocytes, activates GATA-4 (Kitta et al., 2001a) and stimulates interaction between GATA-4 and NFATc (Kakita et al., 2001). However, the functional relationship between GATA-4 and apoptosis in cardiac myocytes has not been tested directly.

The present study examined the effects of anthracyclines on GATA-4 activity in cardiac muscle cells. Our results show that anthracyclines suppress the GATA-4 activity via decreased levels of gene expression. Furthermore, we demonstrate via adenovirus-mediated gene transfer that restoration of GATA activity attenuates apoptosis, directly implicating GATA-4 in regulating cell survival of cardiac muscle cells.

Materials and Methods

Cardiac Muscle Cells. HL-1 mouse cardiac muscle cells (Claycomb et al., 1998) were maintained as described previously (Kitta et al., 2001a).

Cardiac myocytes were isolated from ventricles of adult male Lewis rats (3–6 months old) using an enzymatic isolation technique described previously (Suzuki et al., 1998). Viable myocytes were purified via a series of gravity sedimentation in 0.2, 0.5, and 1 mM Ca^{2+}, and then placed in Dulbecco's modified Eagle's medium/Ham's F-12 medium. Ca^{2+}-tolerant myocytes purified through these procedures were >90% viable.

Electrophoretic Mobility Shift Assay (EMSA). Procedures for nuclear extraction and EMSA have been described previously (Kitta et al., 2001a). The binding reaction mixtures contained 2 μg of protein of nuclear extract, 1 μg of poly(dI-dC), and ^{32}P-labeled double stranded oligonucleotide probe containing consensus sequence for GATA (5′-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3′) in 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 4% (w/v) Ficoll 400, and 20 mM Tris-HCl, pH 7.5.

Supershift experiments were performed by incubating 2 μg of GATA-4, -5, or -6 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in the binding reaction mixture for 1 h at 4°C before the addition of the ^{32}P-labeled oligonucleotide probe to start the binding reaction.

Western Blot Analysis to Monitor GATA-4 Expression. To monitor GATA-4, nuclear extracts were electrophoresed in an SDS-polyacrylamide gel as described previously (Kitta et al., 2001a). The gel was electroblotted onto a polyvinylidene difluoride membrane, and the membrane was blocked and incubated with the polyclonal IgG for GATA-4 (H-112) and actin (H-300) (Santa Cruz Biotechnology, Inc.). The detection was made with horseradish peroxidase-linked secondary antibody and ECL System (Amersham Biosciences Inc., Piscataway, NJ).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA (1 μg) extracted from cells by TRIzol (Invitrogen, Carlsbad, CA) was reverse-transcribed by oligo(dT) priming and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). The resultant cDNA was amplified using *Taq*DNA polymerase (Invitrogen) with a PerkinElmer Gene Amp PCR System 2400, and resolved on a 1.5% agarose gel containing ethidium bromide. PCR primers for murine GATA-4 were designed using Oligo Primer Analysis software: 5′ primer, 5′-GAT GGG ACG GGA CAC TAC CTG-3′; and 3′ primer, 5′-ACC TGC TGG CGT CTT AGA TTT-3′, which produce a 309-base pair product. Denaturing was performed at 94°C for 45 s, annealing for 45 s at 58°C, and polymerase reactions for 2 min at 72°C (25 cycles). The G3PDH mRNA level was also monitored using primers from BD Biosciences Clontech (Palo Alto, CA) as an internal control.

Quantitative PCR was performed using the LightCycler (Roche Diagnostics, Basel, Switzerland). Amplification was carried out using a LightCycler-DNA Master SYBR Green I reaction kit (Roche Diagnostics) and TaqStart antibodies (BD Biosciences Clontech). The amplification program was as follows: 95°C for 30 s, 50 cycles of 95°C for 0 s, 60°C for 5 s, 72°C for 10 s. Fluorescent products were detected at the last step of each cycle. Quantitative analysis of PCR data were performed using the LightCycler Data Analysis software. The level of GATA-4 was normalized to the levels of β-actin.

Northern Blot Analysis. Total RNA (20 μg) was electrophoresed on a 1% agarose gel containing 20 mM MOPS buffer, pH 8.0, 1 mM EDTA and 2.2 M formaldehyde, and transferred onto a ζ-Probe Blotting membrane (Bio-Rad, Hercules, CA). The membrane was optimally cross-linked with UV light and hybridized for 4 h at 62°C with ^{32}P-labeled-specific cDNA probe for GATA-4 (Geneka Biotechnology, Montreal, QC, Canada) in ExpressHyb Hybridization solution (BD Biosciences Clontech). After hybridization, the membrane was washed in 0.1 × standard saline citrate containing 1% SDS, followed by autoradiography.

Adenovirus-Mediated Gene Transfer. Adenovirus expressing GATA-6 was generated using a full-length hGATA-6 clone (Suzuki et al., 1996). Synthetic oligomers were used to subclone a new initiation methionine codon followed by the eight-amino acid coding sequence for the FLAG epitope (DYKDDDDK) and subsequent restoration of the initial 50 base pairs of amino-terminal coding sequence upstream of a unique BssHII restriction site. To generate a control construct, the FLAG-tagged cDNA was digested with *Eco*NI, which cuts at a unique site just upstream of sequences encoding the DNA-binding domain. After fill-in using Klenow polymerase, the plasmid was religated and transformed to create a frame-shifted mutant version of FLAG-hGATA-6. The cDNA inserts for both constructs were subcloned into the pAdTrack-CMV shuttle vector and recombined with pAdEasy-1 using the AdEasy system (He et al., 1998). Correct re-

combinant viruses were identified by restriction mapping and used to generate high-titer virus stocks in 293 cells. Both viruses express green fluorescent protein, but only the wild-type construct makes a functional FLAG-tagged GATA-6 protein. This was tested by Western blot, EMSA, and supershift assays (data not shown). Adenovirus constructs expressing catalase and GATA-4 were kindly provided by Drs. J. Engelhardt (University of Iowa, Iowa City, IA) and J. Molkentin (University of Cincinnati, Cincinnati, OH), respectively.

Adenovirus-directed gene transfer was implemented by adding 100 plaque-forming units of recombinant adenovirus. The culture medium was aspirated from the cell culture growing in a 35-mm dish, and 0.5 ml of the fetal bovine serum-free medium containing the recombinant adenovirus was added. Then 1.5 ml of growth medium was added after 2 h of culture and maintained for 24 to 48 h before performing experiments.

Apoptosis Measurements. The neutral comet assay was used to measure double-stranded DNA breaks as indication of cardiac myocyte apoptosis (Kitta et al., 2001b). Treated cells were embedded in situ in 1% agarose and then placed in lysis solution (2.5 M NaCl, 1% Na-lauryl sarcosinate, 100 mM EDTA, 10 mM Tris base, and 1% Triton X-100) for 30 min. The nuclei were subsequently electrophoresed for 20 min at 1 V/cm, followed by staining with SYBR Green or propidium iodide and visualized with a fluorescence microscope. Between 100 and 150 comets per treatment were scored and assigned into type A, B, or C categories, based on their tail lengths (Krown et al., 1996). Type C comets were defined as apoptotic cells.

To estimate the activities of caspases, which are activated during apoptosis, cleavage of poly(ADP-ribose) polymerase (PARP) was monitored in cell lysates by Western blot analysis as described previously (Kitta et al., 2001b). The polyclonal IgG for cleaved PARP (Cell Signaling Technology Inc., Beverly, MA) was used. The level of 89-kDa cleaved PARP protein was detected with horseradish peroxidase-linked secondary antibody and ECL System.

Statistical Analysis. Means ± S.E. were calculated, and statistically significant differences between two groups were determined by the Student's t test at $p < 0.05$.

Results

Anthracyclines Suppress GATA-4 DNA-Binding Activity. GATA-4 plays a central role in regulating cardiac muscle gene expression. However, the relationship between this transcription factor and anthracycline-induced cardiotoxicity has not been tested directly. Thus, we studied the effects of anthracyclines on GATA-4 DNA-binding activity. Nuclear extracts from HL-1 cardiac muscle cells were found to contain a constitutive binding activity toward the consensus GATA sequence as monitored by EMSA (Fig. 1A, lane 1). This GATA DNA-binding activity was lost in response to treating cells with DNR in a dose-dependent manner (Fig. 1A). The decrease in GATA DNA-binding activity seems specific to the anthracycline pathway because TNFα, another inducer of cell death, had no effect on the levels of GATA-binding activity (Fig. 1A). Indeed, the binding activity was also eliminated by treating the HL-1 cells with another anthracycline compound, DOX (Fig. 1B). Nuclear extracts from the DNR-treated cells are not nonspecifically degraded, because expression levels of actin or total proteins and the DNA-binding activities of SRE and Sp1 were unchanged (Fig. 1C). Furthermore, DNA-binding activities of nuclear factor-κB and Egr-1 were actually increased (Fig. 1C). Primary culture of adult rat ventricular myocytes also responded to DNR, resulting in a reduction of the GATA DNA-binding activity (Fig. 1D). A supershift analysis showed that the majority of the GATA binding protein in HL-1 cells and in adult rat ventricular myocytes is GATA-4, with perhaps a slight contribution from GATA-5 (Fig. 1E). GATA-6 does not seem to contribute to the endogenous DNA-binding activity.

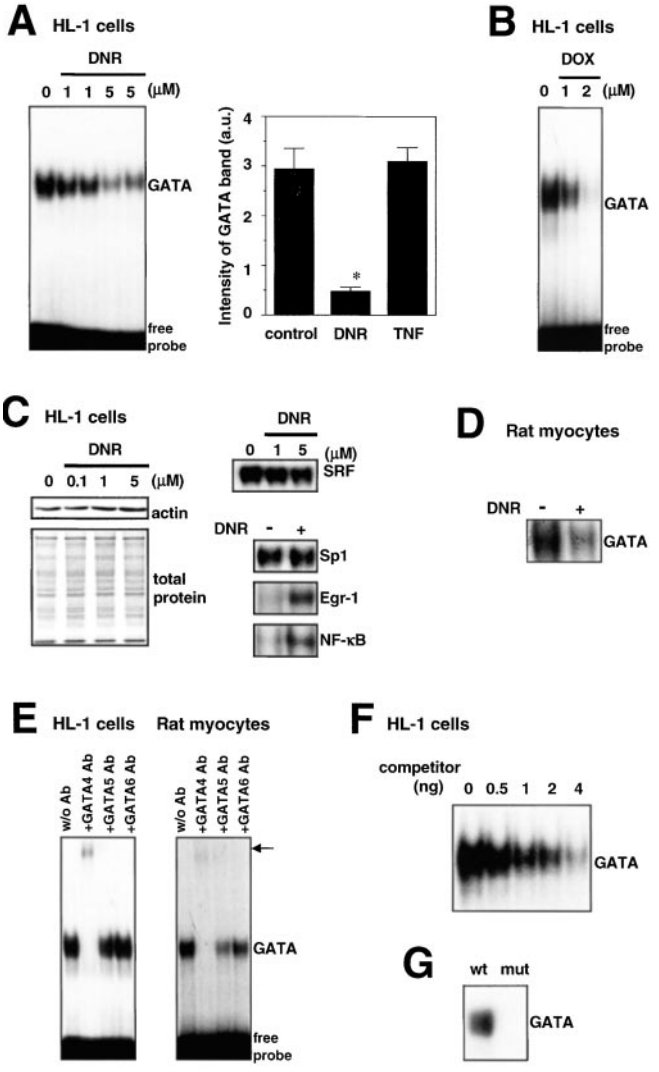

Fig. 1. Anthracyclines decrease levels of GATA-4 activity in cardiac myocytes. A, HL-1 cells were treated with DNR (1 or 5 μM) or TNFα (1 nM) for 24 h. Nuclear extracts were prepared, and the GATA DNA-binding activity was monitored by EMSA. The bar graph represents means ± S.E. of the intensity of the GATA band from untreated and DNR (5 μM)-treated HL-1 cell nuclear extracts as determined by densitometry. *, value is significantly different from the untreated control at $p < 0.05$. B, HL-1 cells were treated with DOX for 24 h. Nuclear extracts were prepared, and the GATA DNA-binding activity was monitored by EMSA. C, HL-1 cells were treated with DNR for 24 h, and the nuclear extracts were prepared. The levels of actin protein were monitored by Western blot, and total protein levels were determined by Coomassie Brilliant Blue staining of the membrane. DNA binding activities of SRF, Sp1, Egr-1, and nuclear factor-κB were monitored by EMSA. D, primary culture of adult rat ventricular myocytes were treated with 2 μM DNR for 24 h. Nuclear extracts were prepared, and the GATA DNA-binding activity was monitored by EMSA. E, extracts from HL-1 cells or adult rat ventricular myocytes were subjected to supershift analysis by incubating EMSA reaction mixtures with the antibodies (Ab) for GATA-4, -5, or -6. The arrow indicates the supershifted bands. F, nuclear extracts from HL-1 cells were subjected to EMSA with ^{32}P-labeled oligonucleotide containing the consensus GATA binding sequence in the presence of increasing amounts of a cold competitor with the GATA consensus sequence in the binding reaction mixtures. G, nuclear extracts from HL-1 cells were subjected to EMSA with ^{32}P-labled oligonucleotide containing the wild-type consensus GATA binding sequence (wt) or a mutated sequence (mut).

Competition of the DNA binding with cold oligonucleotide indicates the specificity of the GATA binding complexes (Fig. 1F). Furthermore, the GATA-4 band was not observed when a ^{32}P-labeled oligonucleotide with the mutated sequence (TCTTA instead of TGATAA) was used (Fig. 1G). These results suggest that anthracyclines can decrease the levels of GATA-4 activity in cardiac myocytes.

GATA-4 Is an Oxidant-Sensitive Transcription Factor. The quinone moiety of the anthracyclines can act as a catalyst for the intracellular formation of ROS (Minotti et al., 1999). Many zinc finger proteins are sensitive to oxidant-mediated inhibition of DNA-binding activity (Webster et al., 2001). Thus, one simple possibility is that anthracyclines generate ROS in the cell, which in turn inhibit the DNA-binding activity of GATA-4. Consistent with this hypothesis, we found that the DNA-binding activity of GATA-4 is oxidant-sensitive, because this is potently inhibited by the in vitro treatment of nuclear extracts with H_2O_2 (Fig. 2A) or a sulfhydryl oxidant, diamide (Fig. 2B). The GATA-4 activity was restored by the addition of a thiol reductant dithiothreitol (data not shown), indicating that oxidants affected the sulfhydryl groups of the GATA-4 molecule. Therefore, anthracycline-mediated inhibition of GATA-4 activity in cardiac muscle cells could be explained by the generation of ROS, in particular H_2O_2, with a subsequent inhibition of the DNA-binding activity.

To determine whether this is indeed the case, we tested the effect of overexpressing the antioxidant enzyme catalase in HL-1 cells. Adenovirus-mediated gene transfer resulted in robust expression of catalase as monitored by Western blot analysis (Fig. 2C, top). The catalase activity increased from 1.2 ± 0.6 nmol $\cdot$ min^{-1} mg^{-1} in control cells to 12.8 ± 1.9 nmol $\cdot$ min^{-1} mg^{-1} in catalase-overexpressing cells. Catalase, however, did not inhibit the DNR-mediated decrease in GATA-4 activity (Fig. 2C). DNR-treatment did not influence the levels of ectopically expressed catalase derived from the adenovirus vector (Fig. 2C, top). The DNR-mediated effect was also unchanged by pretreating the cells with *N*-acetylcysteine, a sulfhydryl reductant and a precursor of glutathione that serves as a cofactor for glutathione peroxidase, another scavenging enzyme of H_2O_2 (Fig. 2D). Therefore, the results indicate that H_2O_2 may not be involved in the cellular actions of anthracyclines on GATA-4. Indeed, in contrast to the in vitro data, treatment of HL-1 cells with H_2O_2 (at concentrations higher than what is expected to be generated by low micromolar anthracycline) did not suppress GATA-4 activity (Fig. 2E). The H_2O_2 exposure to HL-1 cells forms intracellular oxidants as measured using dichlorofluorescein (Kitta et al., 2001c).

Anthracyclines Cause Decreased Levels of GATA-4 Gene Expression. To further evaluate the mechanism of DNR-induced GATA-4 inhibition, Western blot analysis was performed. As shown in Fig. 3A, treatment of HL-1 cells with DNR exhibited a dose-dependent decrease in the levels of nuclear GATA-4 protein, whereas actin expression was unchanged. The time-course study revealed that the DNR effect on levels of GATA-4 protein occurs as early as 8 h and these levels further decline at 24 h (Fig. 3B).

To determine whether the decreased level of GATA-4 protein reflects a translational alteration or deregulated transcription of the gene, RT-PCR analysis was used to monitor the levels of GATA-4 transcripts. The PCR primers for mouse GATA-4 were designed based on the published sequence (Wilson et al., 1993). As shown in Fig. 3C, the GATA-4 mRNA level was decreased in response to a treatment with DNR for 24 h, in a dose-dependent manner. The time-course study revealed that the decrease can be detected as early as 4 h (Fig. 3D). The internal control G3PDH mRNA levels were not altered in response to DNR treatment. These results were also confirmed with a quantitative real-time RT-PCR analysis (Fig. 3E) as well as by Northern blot experiments (Fig. 3F), both of which showed a dose-dependent decrease in levels of the GATA-4 transcript after treatment with DNR. The decrease in GATA-4 mRNA level was also observed, albeit to a lesser extent, in isolated ventricular myocytes from adult rats treated with DNR (2 μM) for 24 h (Fig. 3G).

To determine whether the decreased levels of GATA-4 mRNA in anthracycline-treated cells are caused by an increased rate of mRNA degradation, HL-1 cells were treated with actinomycin D (a general transcription inhibitor) for various time periods after treating cells with DNR. As shown in Fig. 4, a time-dependent decline in the levels of GATA-4 mRNA was detected in the presence of actinomycin D, suggesting that the GATA-4 mRNA was subjected to cellular degradation. However, the rate of GATA-4 mRNA degradation was not increased in the DNR-treated cells. These results indicate that DNR is unlikely to function primarily by altering the stability of GATA-4 mRNA. Rather, the mechanism is likely to be at the transcriptional level.

Effects of Ectopic GATA Expression on Anthracycline-Induced Apoptosis. To test the hypothesis that the anthracycline-mediated decrease in GATA-4 levels is functionally related to the induction of apoptosis, we examined the ability of ectopic GATA transcription factors to rescue DNR-induced apoptosis. Decreased levels of GATA-4 caused by anthracyclines might lead to deregulation of GATA-dependent target genes that are involved in cell survival. As noted above, the majority of the endogenous GATA-binding activity in adult cardiac myocytes is caused by GATA-4. We therefore first tested whether the restoration of GATA-4 expression is sufficient to suppress anthracycline-induced apoptosis, by expressing GATA-4 via adenovirus-mediated gene transfer. HL-1 cells infected with recombinant adenovirus containing the GATA-4 gene (Liang et al., 2001) expressed high levels of GATA-4 in nuclear extracts as monitored by EMSA (Fig. 5A) and in cell lysates as monitored by Western blot analysis (Fig. 5C). Activity of the ectopic GATA-4 was not affected by treating the cells with DNR (Fig. 5A). The comet assay showed that GATA-4 expression attenuated the DNR-induced apoptosis. The incidence of DNR-induced apoptosis in cells infected with GATA-4 expressing adenovirus was significantly less than in cells infected with the control adenovirus (Fig. 5B). Western blotting experiments to analyze levels of cleaved PARP also showed that DNR-induced apoptosis was attenuated by the forced expression of GATA-4, but not by a control adenovirus lacking the GATA-4 gene (Fig. 5C).

To examine whether the suppression of GATA DNA-binding activity may result in the induction of apoptosis, the adenovirus expressing GATA-4-engrailed fusion protein (AdG4-Engr) with dominant negative activity (Liang et al., 2001) was used. As shown in the EMSA results in Fig. 5D, the AdG4-Engr expression caused elimination of the endogenous GATA-4 DNA-binding activity. In contrast, DNA-bind-

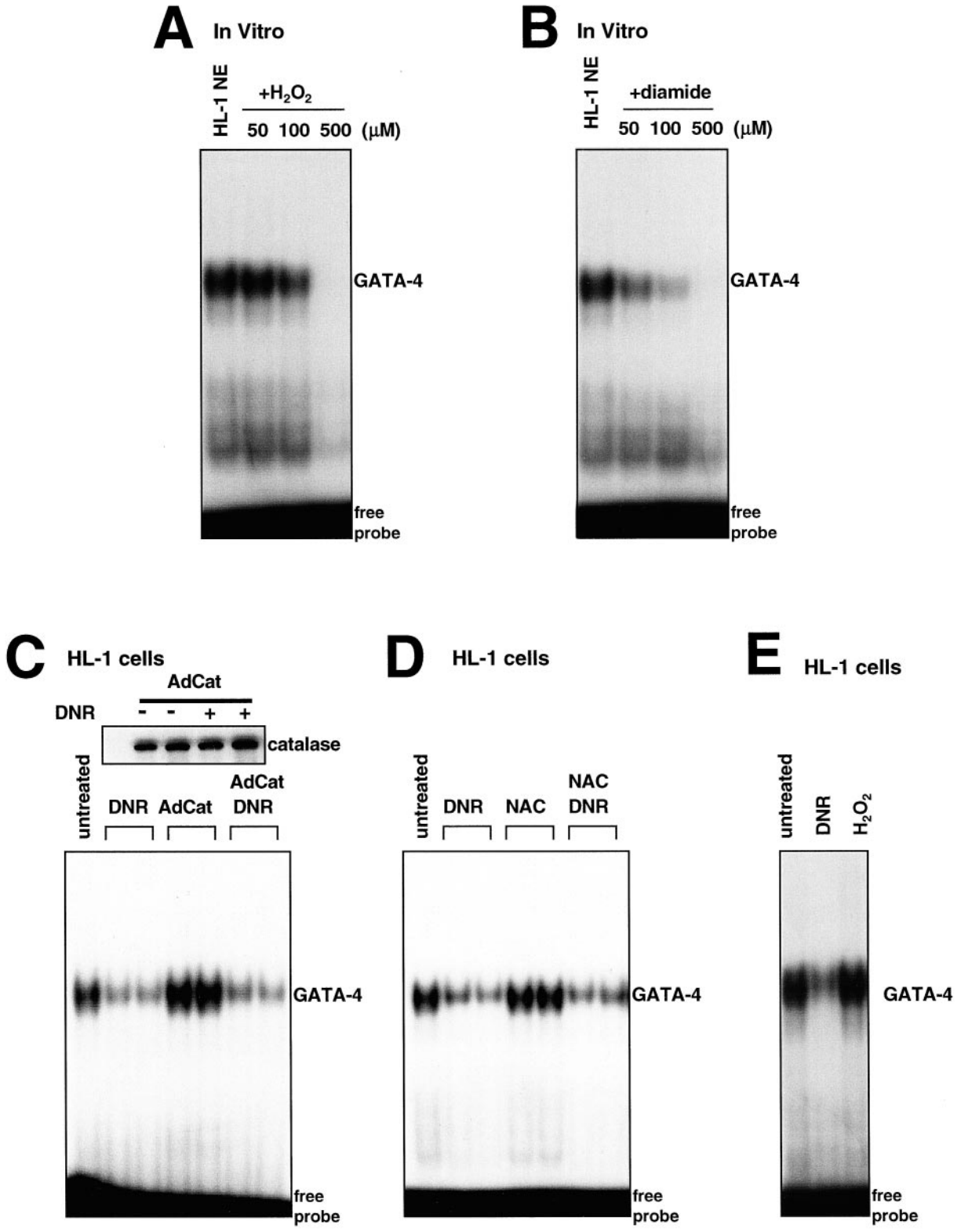

Fig. 2. Role of oxidants in DNR-induced suppression of GATA-4 activity. A and B, nuclear extracts (NE) from HL-1 cells were treated in vitro in the binding reaction mixtures with H_2O_2 (A) or diamide (B) for 1 h before the addition of ^{32}P-labeled oligonucleotide containing GATA consensus sequence, in the absence of dithiothreitol. The DNA-binding activity of GATA-4 was monitored by EMSA. C, HL-1 cells were infected with adenovirus expressing catalase (AdCat) for 48 h and then treated with 5 μM DNR for 24 h. Top, lysates were prepared and the protein levels of catalase were monitored by Western blot. Bottom, nuclear extracts were prepared and the DNA-binding activity of GATA-4 was monitored by EMSA. D, cells were pretreated with *N*-acetylcysteine (NAC; 20 mM) for 4 h, and then treated with 5 μM DNR for 24 h. Nuclear extracts were prepared and the DNA-binding activity of GATA-4 was monitored by EMSA. E, cells were treated with 5 μM DNR or 100 μM H_2O_2 for 24 h. Nuclear extracts were prepared and the DNA-binding activity of GATA-4 was monitored by EMSA.

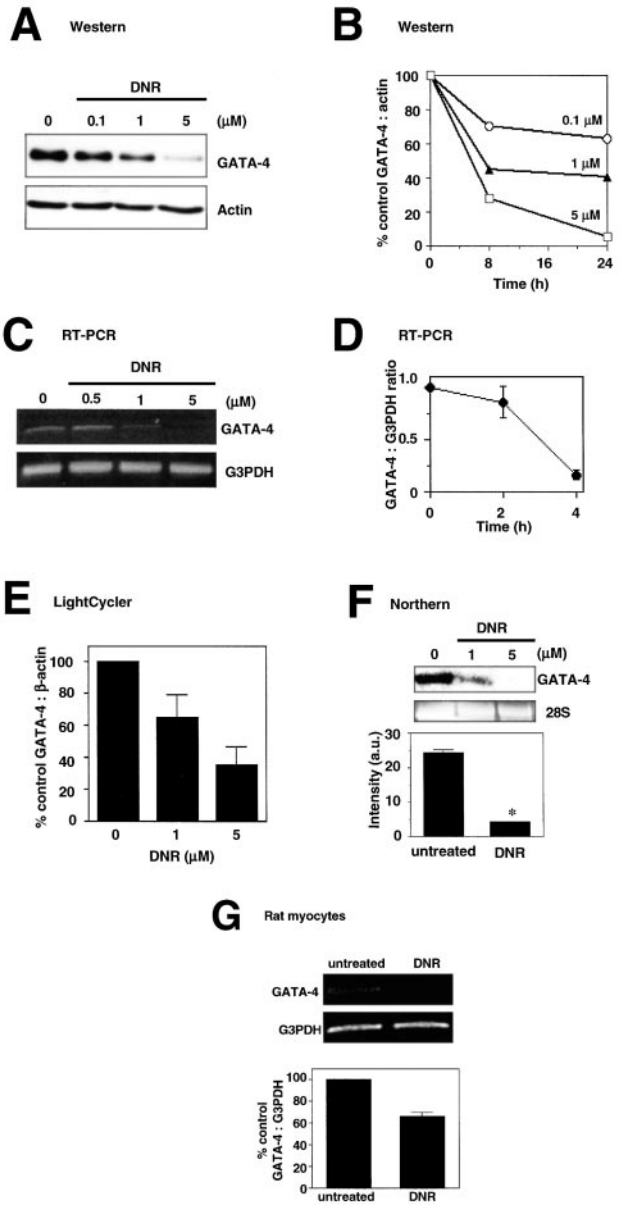

Fig. 3. DNR leads to a reduction in the levels of GATA-4 protein and mRNA. A, HL-1 cells were treated with DNR for 24 h. Nuclear extracts were prepared and the protein levels of GATA-4 and actin were monitored by Western blot. B, line graph indicates the ratio of intensities of GATA-4 to actin proteins as determined by densitometry. C, cells were treated with DNR for 24 h. Total RNA was isolated, and the mRNA levels of GATA-4 and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) were monitored by RT-PCR. D, cells were treated with 2 μM DNR, and the mRNA levels of GATA-4 and G3PDH were monitored by RT-PCR. The line graph indicates means ± S.E. of the ratio of GATA-4 to glyceraldehydes-3-phosphate dehydrogenase bands normalized with the value at time 0. E, cells were treated with DNR for 24 h. Total RNA was isolated and the mRNA levels of GATA-4 and β-actin were monitored by real-time quantitative RT-PCR using a LightCycler. Values represent means ± S.E. of percentage of control of the ratio of the GATA-4 to β-actin levels normalized to the samples from untreated control. F, cells were treated with DNR for 24 h. Total RNA was isolated and the GATA-4 mRNA level was monitored by Northern blot. The levels of 28S RNA as monitored by ethidium bromide staining in an agarose gel are also shown. The bar graph represents means ± S.E. of the intensity of the GATA-4 band. *, value from 5 μM DNR-treated cells is significantly different from the untreated control value at $p < 0.05$. G, primary culture of adult rat ventricular myocytes were treated with 2 μM DNR for 24 h. Total RNA was isolated and the mRNA levels of GATA-4 and glyceraldehydes-3-phosphate dehydrogenase were monitored by RT-PCR. The line graph indicates means ± S.E. of the ratio of GATA-4 to glyceraldehydes-3-phosphate dehydrogenase bands.

ing activities of other transcription factors such as Sp1 were not altered by this dominant negative mutant (data not shown). Forty-eight hours after infection, the levels of cleaved PARP significantly increased, indicating that a suppression of GATA-4 DNA-binding activity alone can result in apoptosis.

To determine whether the cell survival effect in cardiac myocytes is specific to GATA-4, the ability of GATA-6 to rescue apoptosis was tested. As indicated in Fig. 1E, HL-1 cells or adult rat ventricular myocytes do not normally express significant levels of this transcription factor. However, we found that adenovirus-mediated expression of GATA-6 also attenuated apoptosis induced by DNR. As shown in Fig. 6A, adenovirus-mediated gene transfer was capable of generating high levels of GATA-6 expression in HL-1 cells, as monitored by Western blotting and EMSA experiments. Similar to what we observed with ectopic GATA-4, DNR did not influence ectopic GATA-6 activity (data not shown). Forced expression of GATA-6 attenuated the DNR-induced apoptosis as monitored by the comet assay (Fig. 6B). A control adenovirus expressing a frame shifted (non-DNA-binding) GATA-6 protein did not attenuate the apoptosis. These results were confirmed by Western blot monitoring PARP cleavage as shown in Fig. 6C. A densitometry analysis indicated statistically significant attenuation of DNR-induced apoptosis by GATA-6 expression. To confirm that a similar mechanism functions in primary cells, comet assays were performed on cultures of isolated rat ventricular myocytes. Myocytes infected with control or GATA-6 expressing adenovirus were selected based on coexpression of GFP. As shown

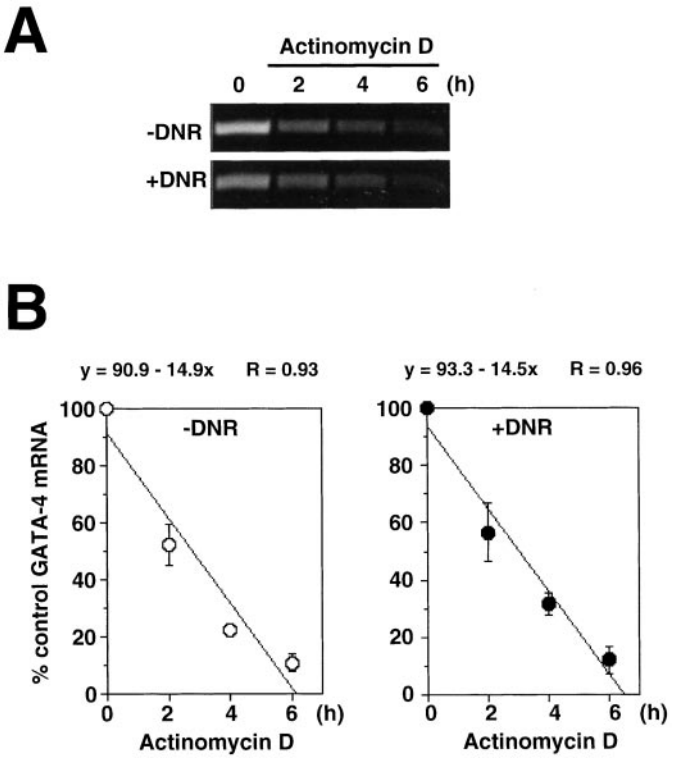

Fig. 4. Effects of DNR on GATA-4 mRNA stability. HL-1 cells were treated with 1 μM DNR for 24 h and then incubated with 5 μg/ml actinomycin D for durations indicated. Total RNA was isolated and the levels of GATA-4 mRNA were monitored by RT-PCR. A, representative results. B, densitometry analysis. Values represent means ± S.E. of percentage of control relative to values without actinomycin D treatment.

in Fig. 6D, forced expression of GATA-6 was sufficient to provide a significant attenuation of DNR-induced apoptosis in primary myocyte cells.

Discussion

Our results show that anthracyclines induce cardiac myocyte apoptosis and decrease the levels of GATA-4 activity. Because TNFα, which predominantly induces apoptosis via the death receptor-mediated mechanism, does not alter the levels of GATA-4, the changes caused by anthracyclines are not a nonspecific event associated with cell death. Furthermore, restoration of GATA activity by ectopic expression attenuates apoptosis, indicating that decreased levels of GATA-4 may mediate the induction of apoptosis. These novel results provide direct evidence linking GATA-4 and suppression of apoptosis in cardiac myocytes. Cardiac myocyte loss and subsequent decline of cardiac function is a major problem in anthracycline-induced cardiotoxicity. Thus, our findings have significance for understanding the molecular mechanisms of the actions of cancer chemotherapeutic agents on the heart.

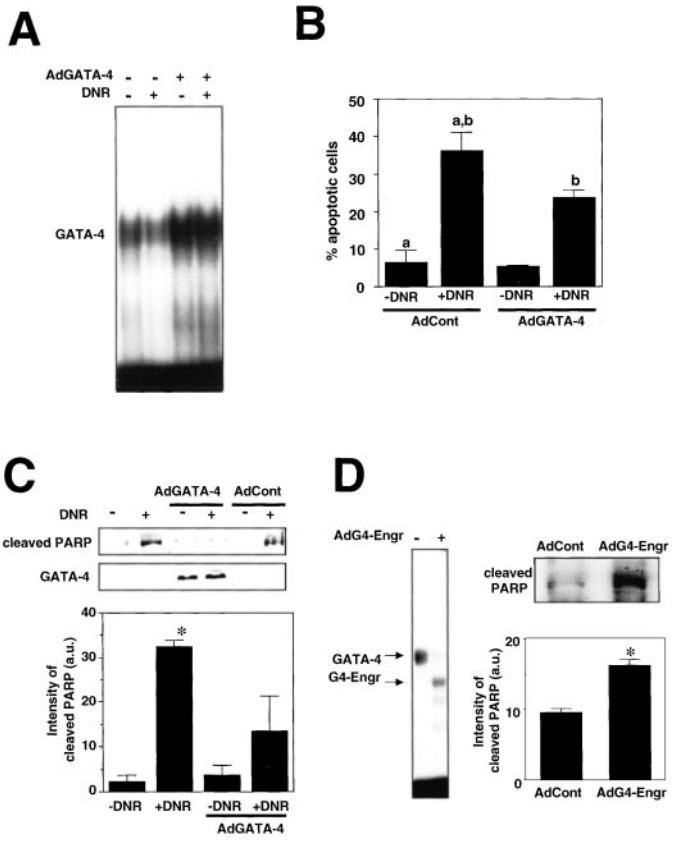

Fig. 5. Effects of forced GATA-4 expression and a dominant negative mutant on apoptosis. A, HL-1 cells were infected with adenovirus expressing wild-type mouse GATA-4 (AdGATA-4) for 48 h, and then treated with 2 μM DNR for another 24 h. Nuclear extracts were subjected to EMSA. B, cells were infected with adenovirus expressing GATA-4 or control adenovirus (AdCont), and then treated with DNR. Incidence of apoptosis was determined by neutral comet assay. The values represent means ± S.E. of percentage of apoptotic cells. a and b indicate that the values with the same letter are significantly different from each other at $p < 0.05$. C, cells were infected with adenovirus expressing GATA-4 or control adenovirus, and then treated with DNR. Cell lysates were prepared and subjected to Western blot analysis to monitor the levels of cleaved PARP (top). The values in the bar graph represent means ± S.E. of the intensity of cleaved PARP bands ($n = 4$). *, value is significantly different from the untreated control at $p < 0.05$. The membrane was stripped and reblotted with the GATA-4 antibody. Unlike nuclear enriched fractions, in cell lysates, the endogenous GATA-4 cannot be observed because of the sensitivity of the antibody (Kitta et al., 2001a). D, cells were infected with adenovirus expressing GATA-4-engrailed fusion protein (AdG4-Engr). EMSA results show that AdG4-Engr effectively eliminated the GATA-4 DNA binding activity in nuclear extracts. Western blot was used to monitor PARP cleavage. The values in the bar graph represent means ± S.E. of the intensity of cleaved PARP bands ($n = 4$). *, value is significantly different from the value from control adenovirus-infected cells at $p < 0.05$.

Implications to Cancer Chemotherapy. The anthracyclines are effective cancer chemotherapeutic agents; however, they can exert severe cardiotoxicity. Thus, attenuating cardiotoxic actions of anthracyclines is expected to have a tremendous impact on the treatment of cancer. Recent data demonstrated that anthracyclines induce cardiac myocyte apoptosis (Arola et al., 2000; Kang et al., 2000) and decrease the levels of antiapoptotic protein Bcl-x_L (Negoro et al., 2001), which is regulated by GATA factors (Gregory et al., 1999). Our laboratory recently found that forced expression of GATA-4 enhances the expression of Bcl-x_L in cardiac myocytes (Kitta et al., 2003). Anthracyclines also suppress expression of several GATA-regulated cardiac genes such as cardiac adriamycin-responsive protein (Jeyaseelan et al., 1997), brain and atrial natriuretic proteins (Chen et al., 1999), α-myosin heavy chain (Saadane et al., 1999), and calsequestrin (Arai et al., 1998). The present study suggests that modulating the levels of GATA factors may be an effective therapeutic strategy against anthracycline-induced cardiotoxicity.

Mechanism of GATA-4 Down-Regulation. Because anthracyclines have been shown to exert oxidative stress, we considered that this might influence directly the DNA-binding activity of GATA-4. GATA-4 is a zinc finger transcription factor, and many zinc finger DNA-binding proteins are inhibited by oxidation (Webster et al., 2001). Consistent with this hypothesis, our in vitro experiments indicated that GATA-4 is oxidative stress-sensitive, because oxidants such as H_2O_2 can suppress its DNA-binding activity. This in vitro effect probably involves oxidation of the critical zinc-coordinating sulfhydryl groups, because diamide (a more specific sulfhydryl oxidant) also inhibited GATA-4 DNA-binding activity, whereas the sulfhydryl reductant dithiothreitol restored the activity. However, our cellular studies showed that direct sulfhydryl oxidation cannot explain DNR-induced inhibition of GATA-4 activity, because whole cell treatment with H_2O_2 did not lead to an inhibition of the GATA-4 activity. In fact, the results using forced expression of catalase indicate that suppression of GATA-4 gene expression is independent of ROS. The mechanism of inhibition acts instead at the level of GATA-4 gene expression.

In Western and Northern blotting experiments, we found that anthracyclines inhibit GATA-4 protein expression via suppressing the production of GATA-4 mRNA. Experiments using actinomycin D, a general inhibitor of gene transcription, determined that DNR did not alter the stability of GATA-4 mRNA. These results all support a mechanism by which anthracyclines inhibit transcription of the GATA-4 gene. Further insight into the signal transduction mechanism that suppresses GATA-4 transcription may come from analysis of the GATA-4 promoter region and identification of specific *cis*-elements that mediate the inhibition (or the loss of an activator).

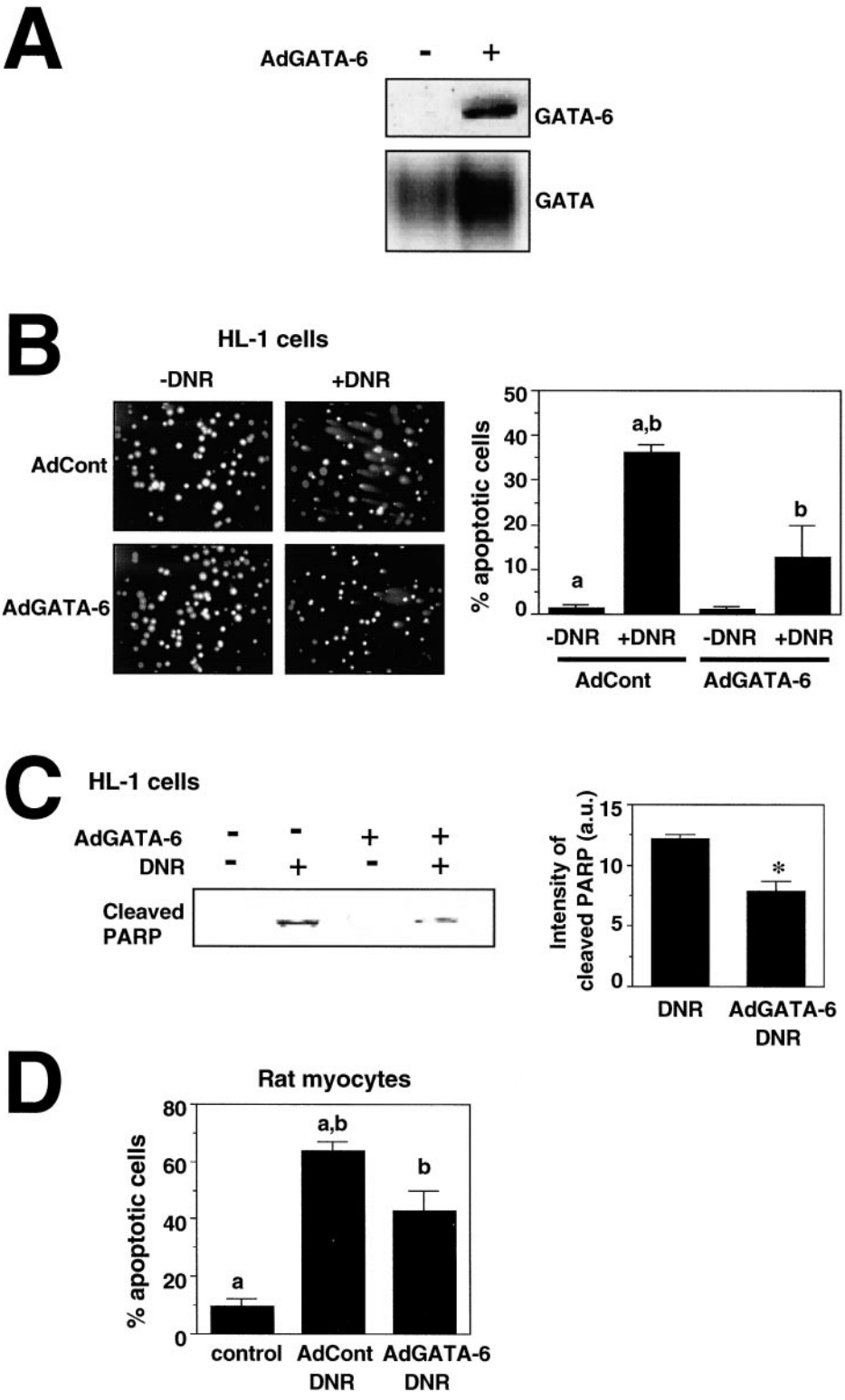

Fig. 6. Effects of forced GATA-6 expression on DNR-induced apoptosis. A, HL-1 cells infected with adenovirus expressing wild-type GATA-6 (AdGATA-6) for 48 h. Nuclear extracts were prepared and GATA-6 expression was monitored by Western blotting experiments (top), and GATA DNA-binding activity was monitored by EMSA (bottom). B, HL-1 cells were infected with adenovirus expressing GATA-6 or control adenovirus (AdCont) and then treated with DNR. Incidence of apoptosis was determined by neutral comet assay. The left panels show representative results. The values in the bar graph represent means ± S.E. of percentage of apoptotic cells. a and b indicate that the values with the same letter are significantly different from each other at $p < 0.05$. C, HL-1 cells were infected with adenovirus expressing wild-type GATA-6 for 48 h and then treated with DNR for another 24 h. Cell lysates were prepared and the level of cleaved PARP was monitored by Western blot. Values in the bar graph represent means ± S.E. of the intensity of cleaved PARP band as determined by densitometry analysis ($n = 4$). *, value is significantly different at $p < 0.05$. D, primary culture of adult rat ventricular myocytes were infected with adenovirus expressing GATA-6 or control adenovirus, then treated with DNR. Incidence of apoptosis was determined by neutral comet assay. The values in the bar graph represent means ± S.E. of percentage of apoptotic cells. a and b indicate that the values with the same letter are significantly different from each other at $p < 0.05$.

Suppression of GATA-4 Expression as a Mechanism of Cardiac Myocyte Apoptosis. We previously reported that anthracycline induces apoptosis of isolated adult rat cardiac myocytes and of HL-1 cells (Kitta et al., 2001b). The present data, showing that anthracyclines lead to a suppression of GATA-4 expression, indicates that GATA-4 may normally inhibit apoptotic events. A number of antiapoptotic genes have GATA elements in their promoter regions (O'Prey et al., 1993; Zhang et al., 1995; Grillot et al., 1997; Keinanen et al., 1999), and GATA-1 has been shown to regulate apoptotic signaling (Blobel et al., 1995; Weiss and Orkin, 1995; Blobel and Orkin, 1996; Gregory et al., 1999). Both GATA-4 and GATA-6 were implicated previously as cell survival factors in different noncardiac cell types (Heikinheimo et al., 1997; Ghatpande et al., 2000; Shureiqi et al., 2002). To provide direct evidence that GATA-4 regulates cardiac myocyte apoptosis, GATA transcription factors were expressed ectopically before cells were treated with DNR. Using adenovirus-mediated gene transfer, it was possible to bypass the inhibition of GATA-4 transcription. We showed that ectopic expression of either GATA-4 or GATA-6 was sufficient to attenuate the incidence of apoptosis, strongly indicating that GATA-4 is a cell survival mediator. Furthermore, a suppression of the GATA-4 activity by the dominant negative mutant (Liang et al., 2001) induced the apoptotic cell death. Thus, the decreased levels of this factor may be responsible for the induction of apoptosis by anthracyclines, although GATA-directed repression by engrailed domain may decrease the activity of other transcription factors (Han and Manley, 1993).

In summary, the present study demonstrates that anthracyclines decrease the levels of GATA-4 and provides, for the first time, evidence that GATA-4 controls apoptotic events in cardiac myocytes. Here, we have used primarily the HL-1 cardiac muscle cell line as a model for differentiated cardiomyocytes, although the results are consistent also with the analysis of primary rat ventricular myocytes, which is limited for technical reasons. Our data indicate that GATA-4 mediates cell survival, and a decrease in levels of GATA-4 may in fact serve to trigger the induction of apoptosis. Therapeutic strategies that increase the activity of GATA-dependent gene transcription, such as forced expression of GATA factors via genetic modulation and/or activation of GATA-4 via humoral agents, may have beneficial effects toward anthracycline-induced cardiotoxicity where the loss of cardiac myocytes may pose a significant problem. These therapies, however, must be pursued with caution because increased activities of GATA transcription factors could exert unwanted clinical manifestations such as cardiac hypertrophy (Liang et al., 2001). The fact that GATA-6 can replace GATA-4 to regulate cell survival indicates that therapies need not rely specifically on GATA-4 but might exploit other GATA factors, which might avoid side-effects. Further research is needed to understand the cell biology of GATA transcription factors in the heart to modulate these factors for the prevention of anthracycline-induced cardiomyopathy.

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