Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity

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In recent years, significant progress has been made in understanding cardiomyocyte differentiation. However, little is known about the regulation of myocyte survival despite the fact that myocyte apoptosis is a leading cause of heart failure. Here we report that transcription factor GATA-4 is a survival factor for differentiated, postnatal cardiomyocytes and an upstream activator of the antiapoptotic gene *Bcl-X*. An early event in the cardiotoxic effect of the antitumor drug doxorubicin is GATA-4 depletion, which in turn causes cardiomyocyte apoptosis. Mouse heterozygotes for a null *Gata4* allele have enhanced susceptibility to doxorubicin cardiotoxicity. Genetic or pharmacologic enhancement of GATA-4 prevents cardiomyocyte apoptosis and drug-induced cardiotoxicity. The results indicate that GATA-4 is an antiapoptotic factor required for the adaptive stress response of the adult heart. Modulation of survival/apoptosis genes by tissue-specific transcription factors may be a general paradigm that can be exploited effectively for cell-specific regulation of apoptosis in disease states.

transcription | apoptosis | Bcl-X | doxorubicin | α1-adrenergic receptors

Over the past years, a great deal has been learned about the mechanisms of cardiomyocyte differentiation, heart development, and pathologic myocyte growth (1). Much less is understood with respect to the regulation of myocyte survival. Unlike other organs, the heart has a limited regenerative potential, and myocyte loss is a determining factor of cardiac dysfunction (CD), the leading cause of mortality in industrialized countries. It is now well established that myocyte death occurs largely through apoptosis (2–4). However, and despite the obvious relevance to human disease, the mechanisms that regulate apoptosis of differentiated cardiomyocytes remain poorly understood.

Apoptosis, or programmed cell death, is an evolutionarily conserved mechanism, allowing the removal of damaged or unwanted cells from the organisms through activation of a set of proteases known as caspases (5). Caspase activation can be triggered either by engagement of cell surface "death receptors" (6) or by cellular stress (7, 8). The Bcl-2 family of proteins are key regulators of the stress-induced apoptotic pathway (9), and alterations in the ratio of pro-/antiapoptotic Bcl-2 family members affect myocyte survival and have been observed in a variety of experimental and clinical settings (10, 11). These changes often occur at transcriptional levels, yet little is known regarding transcriptional regulation and upstream activators of the *Bcl-2* gene family.

Myocyte apoptosis can be induced by numerous stimuli including ischemia, proinflammatory cytokines, and antitumor treatments (10). In fact, cardiac toxicity caused by myocyte apoptosis is a major complication of several chemotherapeutic regimens, especially those involving anthracyclines, which are among the most effective and widely used antitumor agents (12, 13). Several lines of evidence suggest that the progressive anthracycline-induced cardiomyopathies result from an initial subtle effect on the myocyte-differentiation program that alters myocyte survival and, ultimately, the adaptive response of the heart. Genes with expression that is inhibited by anthracyclines include sarcomeric genes, genes encoding energy-producing enzymes, and transcription factors (14–17).

Because identification of the nuclear effectors of anthracyclines may provide insight into the regulatory pathways of myocyte survival, we set out to characterize the transcriptional mechanisms underlying anthracycline action in the heart. The results reveal that GATA-4, a key regulator of heart development (18–21), is an essential survival factor for postnatal cardiomyocytes and is depleted rapidly in response to doxorubicin (Dox) treatment. The action of GATA-4 on cell survival involves direct regulation of the apoptotic program via transcriptional activation of $Bcl\text{-}X_L$. The work suggests that targeting tissue-specific transcription factors may be an effective approach to cell-specific regulation of apoptosis.

Methods

Cell Cultures. All procedures with neonatal cardiomyocytes including RNA and protein extractions and analysis were as described (19). Dox was used at 300 nM and phenylephrine (Phe) at 100 nM (Sigma). The Bcl-X antibody was purchased from Santa Cruz Biotechnology.

DNA Vectors. Atrial natriuretic factor (ANF) and Rous sarcoma virus (RSV) reporter plasmids as well as GATA-4 expression vectors and recombinant adenoviruses were described previously (20, 22). The Bcl-X-luc reporter was a kind gift of Harvey F. Lodish (Massachusetts Institute of Technology, Cambridge) (23).

***In Vivo* Experiments.** Mice (C3H, 60–90 days old, Charles River Breeding Laboratories) were handled in accordance with institutional guidelines. Experiments were approved by the Animal Ethics Committee of Institut de Recherches Cliniques de Montréal. The $Gata4^{+/-}$ mice were described in ref. 24. Mice were treated with 15 mg/kg Dox i.p., saline with or without s.c. infusion of Phe (1 mg/kg per day), or vehicle (saline containing 0.002% ascorbic acid) by using Alzet microosmotic pump model 1002 (Durect Corporation, Cupertino, CA). After the indicated time period, the mice were anesthetized with 12–15 μl/g i.p Avertin (2.5% solution) and killed. Systolic blood pressure was determined by the tail-cuff technique (25). The day before echocardiography, the mice were anesthetized (1.5% isoflurane, 75 ml/min 100% O_2), and their anterior chests were shaved. Two-dimensional guided M-mode echocardiography was performed under conscious sedation with 15 μl/g i.p. of a 1:1 mixture of fentanyl (5 μg/ml) and droperidol (250 μg/ml) by using a Hewlett–Packard Sonos 5500 and a 15-MHz linear-array

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Abbreviations: CD, cardiac dysfunction; Dox, doxorubicin; Phe, phenylephrine; ANF, atrial natriuretic factor; FS, fractional shortening; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP end-labeling; SRE, serum response element.

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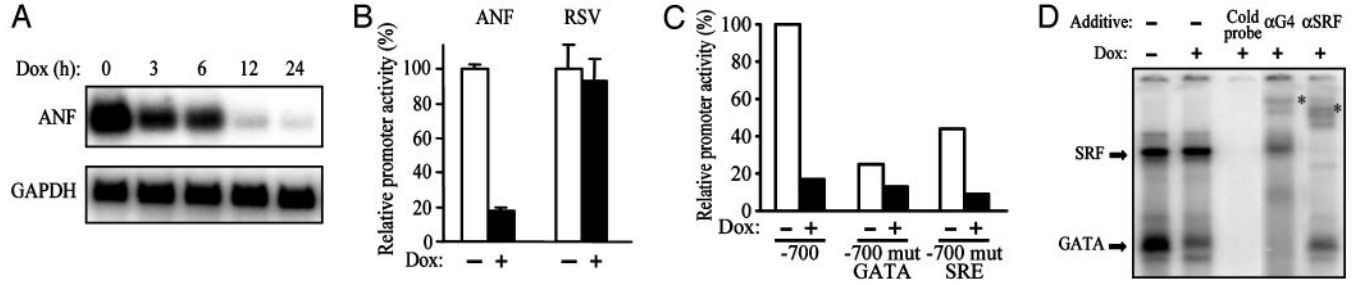


Fig. 1. Identification of a Dox response element on the ANF gene. (*A*) Northern blot analyses of total RNA from primary cardiomyocyte cultures treated with Dox for the indicated time period. (*B* and *C*) Inhibition of ANF promoter activity by 12 h of treatment with Dox (19). The −700mutGATA construct contains point mutations in the three GATA-binding sites. The −700mutSRE construct contains point mutations in the proximal SRE. The results shown represent the mean ± SEM of at least six independent determinations. RSV, Rous sarcoma virus. (*D*) Gel-shift analyses with nuclear extracts prepared from control or Dox-treated cardiomyocytes and the GATA/SRE probe. Supershift (indicated by asterisks) with the serum response factor (αSRF) and GATA-4 (αG4) antibodies was used to confirm the identity of each complex.

transducer. The mice were secured lightly in the left lateral decubitus position on a warming pad to maintain normothermia. To improve the near-field image for visualization of the interventricular septum, a standoff for the transducer tip was used. A 3- to 5-mm-thick 1% agarose pad was inserted between the chest of the mice and the transducer. The montage was sealed acoustically with prewarm acoustic coupling gel. Fractional shortening (FS) and left ventricular weight were determined as described (26). CD is defined as FS $< 45\%$.

Immunohistochemistry and Terminal Deoxynucleotidyltransferase-Mediated dUTP End-Labeling (TUNEL). Immunohistochemistry was performed as described (27). Apoptosis was detected by the TUNEL technique as recommended in the Apoptag kit (Intergen, Purchase, NY) and counterstained with methyl green. An average of 10 random fields with 100–200 nuclei per field was analyzed.

Statistics. The data are reported as mean ± SEM or frequency ratio. A Student's unpaired *t* test was used to compare two groups. Multiple group comparisons were made by using the one-way ANOVA test followed by the Student–Newman–Keuls test or a repeated-measures ANOVA test followed by the protected-*t* Fisher's least-squares difference test. Frequencies were analyzed by using a contingency table and the χ^2 independence test. Rate of survival was determined by using a Kaplan–Meier survival analysis followed by a generalized Wilcoxon test to test for equality of survival. In all cases, differences were considered to be statistically significant when $P < 0.05$.

Results

GATA-4 Is a Survival Factor for Postnatal Cardiomyocytes. ANF is the major heart secretory product and an essential regulator of cardiovascular homeostasis (28). ANF transcription is highly regulated, and molecular dissection of the ANF promoter has contributed greatly to our present knowledge of cardiac transcription and cardiomyocyte differentiation (29).

Transcription of the *ANF* gene is severely inhibited by Dox treatment of primary cardiomyocyte cultures (Fig. 1 *A* and *B*). This effect is elicited rapidly (50% decrease) after only 3 h of exposure to Dox (the earliest time examined) and peaks at 12 h. Mutational analysis (Fig. 1*C*) revealed that Dox essentially targets the proximal ANF promoter, and maximal Dox response requires intact binding sites for the GATA-4 transcription factor; mutation of these sites significantly reduces the response to Dox, whereas mutation of the serum response element (SRE) (30) reduces basal promoter activity but maintains Dox responsiveness. Mutations or deletions of other cis regulatory elements that alter basal promoter activity, including AP-1 (31) and the distal SRE (32), had no effect on Dox responsiveness (data not shown). Together, these findings identified a nuclear pathway for Dox regulation of cardiac transcription and suggested that GATA-4 might be a major nuclear target for Dox. This conclusion was supported further by the finding that GATA-4 but not serum response factor binding to the Dox response element was decreased dramatically in nuclear extracts prepared from Dox-treated cardiomyocytes (Fig. 1*D*). RNA and protein analysis confirmed that Dox treatment in mice and isolated primary cardiomyocyte cultures causes rapid depletion of GATA-4 (Fig. 2). The negative effect of Dox on GATA-4 transcripts was abrogated by actinomycin D, an inhibitor of RNA polymerase

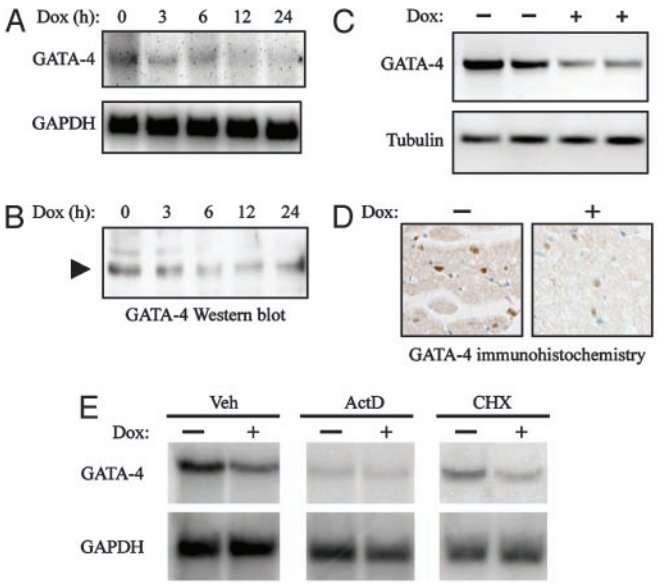


Fig. 2. Rapid depletion of GATA-4 transcripts and proteins after Dox treatment *in vitro* (*A*, *B*, and *E*) and *in vivo* (*C* and *D*). Total RNA (*A*) and nuclear extracts (*B*) were prepared from primary cardiomyocyte cultures treated for the indicated times with Dox and subjected to Northern and Western blot analyses, respectively. The arrowhead indicates GATA-4. Note how GATA-4 mRNA is already depleted after 3 h of Dox treatment. (*C* and *D*) Seven days after treatment with or without Dox, total RNA or tissue sections were prepared from the heart and assessed by semiquantitative RT-PCR (*C*) and immunohistochemistry (*D*). Similar decreases in GATA-4 mRNA or proteins were systematically observed in all animals treated with Dox (six to eight per group). (*E*) Northern blot analysis using total RNA from cardiomyocytes treated with vehicle (Veh), 5 mg/liter actinomycin D (ActD), or 10 μM cycloheximide (CHX) for 6 h. Dox-induced inhibition of GATA-4 mRNA is abrogated in the presence of actinomycin D but not cycloheximide.

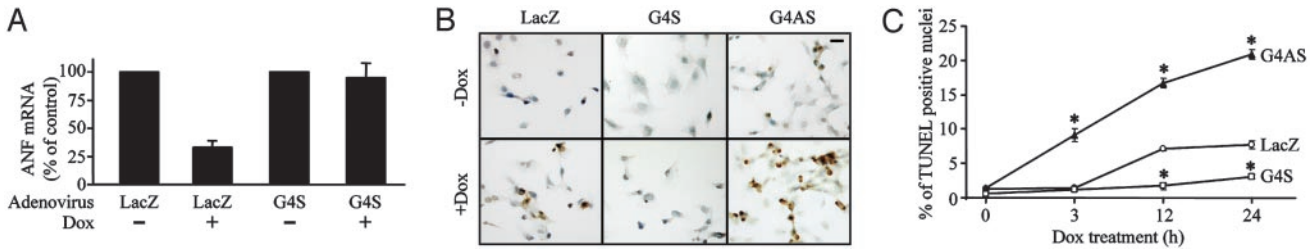


Fig. 3. GATA-4 protects cardiomyocytes against Dox cardiotoxicity. GATA-4 levels were manipulated by using adenovirus-mediated transfer of GATA-4 sense (G4S) or antisense (G4AS) transcripts (20). (*A*) Northern blot quantification of changes in ANF mRNA in cardiomyocytes infected with control (LacZ) or G4S adenovirus vectors. Cells were harvested after 12 h of treatment. The data represent the mean ± SEM (three in each group). (*B*) The contribution of GATA-4 to myocyte apoptosis was assessed 24 h after Dox treatment by using TUNEL assays. (Scale bar, 20 μm.) (*C*) Quantification of TUNEL assays in cardiomyocytes with altered GATA-4 levels. The data represent the mean ± SEM (three per group; *, $P < 0.01$ vs. LacZ).

II-dependent transcription, but not by cycloheximide (Fig. 2*E*). Thus, *GATA-4* seems to be an immediate early-response gene to Dox.

GATA-4, a member of the zinc finger family of GATA proteins, is a key regulator of the cardiac genetic program (18–20). GATA-4 also is required for the adaptive response of cardiomyocytes (20, 33, 34). To determine the role of Dox inhibition of GATA-4 on Dox cardiotoxicity, we tested the consequences of increased GATA-4 activity on Dox effects. First, we used adenovirus-mediated gene transfer to overexpress sense or antisense GATA-4 transcripts in cultured cardiomyocytes. Consistent with our previous results (20), infection of cardiomyocytes with the antisense vector resulted in >80% reduction of GATA-4 protein, whereas the sense vector increased protein levels by 2- to 3-fold. Overexpression of GATA-4 significantly attenuated Dox-induced down-regulation of the *ANF* gene (Fig. 3*A*) as well as Dox-induced myocyte apoptosis, as evidenced by the absence of TUNEL-positive nuclei (Fig. 3 *B* and *C*). In contrast, cells infected with the GATA-4 antisense vector showed decreased survival in the absence of Dox and an exaggerated response to Dox-induced apoptosis, which was evidenced by the significant increase of TUNEL-positive cells at earlier time points as well as a 3-fold increase in apoptotic cells at later time points (Fig. 3*C*).

To confirm *in vivo* the link between GATA-4 depletion and myocyte apoptosis as well as CD in response to Dox, we analyzed the cardiac response to a single injection of Dox in mice heterozygous for a null allele of the *Gata4* gene and in their wild-type littermates (*Gata4* null mice are embryonic-lethal and therefore could not be tested). At baseline, *Gata4*$^{+/-}$ mice had similar FS as their wild-type littermates (59% vs. 57.9%). However, although cardiac function was not altered significantly by Dox treatment in wild-type mice (only one animal showed reduced FS), 75% of *Gata4*$^{+/-}$ mice showed significantly decreased cardiac function, bringing the mean FS of the *Gata4*$^{+/-}$ mice to 43% vs. 53% in the wild-type group ($P < 0.05$) (Fig. 4). Given the deteriorating cardiac function of the *Gata4*$^{+/-}$ mice, the protocol was terminated at this stage, and animals were killed to determine the rate of myocyte loss in each group. TUNEL assays revealed a 2-fold increase in the percentage of apoptotic cardiomyocyte nuclei in *Gata4*$^{+/-}$ mice (0.93 ± 0.12 vs. 0.45 ± 0.004; $P < 0.01$). Thus, both the *in vitro* and *in vivo* data reveal a critical role for GATA-4 in the survival of terminally differentiated cardiomyocytes in response to cardiac stress and suggest that Dox cardiotoxicity is caused by interference with this pathway.

GATA-4 Is a Regulator of the Antiapoptotic Gene *Bcl-X*$_L$. To elucidate the specific target(s) of GATA-4 in the apoptotic pathway, we used DNA microarrays to analyze changes in gene expression in cardiomyocytes in which GATA-4 levels were manipulated. These studies revealed that a 2-fold increase in GATA-4 levels was associated with up-regulation of Bcl-X$_L$ transcripts, a result that was confirmed further by using real-time quantitative PCR (Fig. 5*A*) as well as Western blot analysis (Fig. 5*B*). Interestingly, Dox treatment also was associated with marked down-regulation of the antiapoptotic Bcl-X$_L$ protein, and overexpression of GATA-4 protected against Dox-induced Bcl-X$_L$ depletion (Fig. 5*B*). These results suggested that the cardioprotective effect of GATA-4 may be due in part to direct modulation of the *Bcl-X* gene. Two consensus GATA-binding sites are present within the 5′ region of the *Bcl-X* gene (Fig. 5*C*), upstream of the major transcription-initiation sites (35). In gel-shift assays, oligonucleotide probes corresponding to these sites effectively compete for GATA-4 binding (Fig. 5*C Top*) with the proximal GATA site displaying greater affinity compared with the distal site. Moreover, both sites are able to directly bind recombinant or endogenous GATA-4 present in cardiomyocyte extracts (Fig. 5*C Middle*), suggesting that *Bcl-X* may be a direct target for GATA-4. This interaction was confirmed further in cotransfection assays in which GATA-4 dose-dependently activated a Bcl-X-luc reporter (23) up to 6-fold (Fig. 5*D*). Moreover, Bcl-X promoter activity was decreased by 5-fold in cardiomyocytes treated with Dox (Fig. 5*E*), showing a direct correlation between GATA-4 levels and Bcl-X transcription. Together these data establish GATA-4 as an upstream activator of *Bcl-X*$_L$.

Pharmacologic Modulation of GATA-4. The results obtained above by using genetic modulation of GATA-4 indicated that maintenance of GATA-4 activity was crucial for myocyte survival and suggested that GATA-4 activators may function as cardioprotectors. Very few upstream activators of GATA-4 are known,

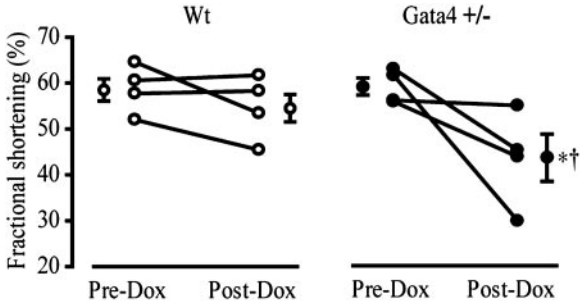


Fig. 4. A 50% reduction in GATA-4 levels exacerbates Dox cardiotoxicity *in vivo*. FS was determined by using echocardiography in *GATA-4*$^{+/-}$ mice and their wild-type (Wt) littermates before (Pre-Dox) and 1 week after (Post-Dox) Dox treatment. FS of the *GATA-4*$^{+/-}$ mice was reduced significantly after Dox treatment and as compared with the wild-type group. The data represent each individual mouse FS and mean ± SEM (three per group; *, $P < 0.05$ vs. pre-Dox wild type; †, $P < 0.05$ vs. pre-Dox *GATA-4*$^{+/-}$ mice).

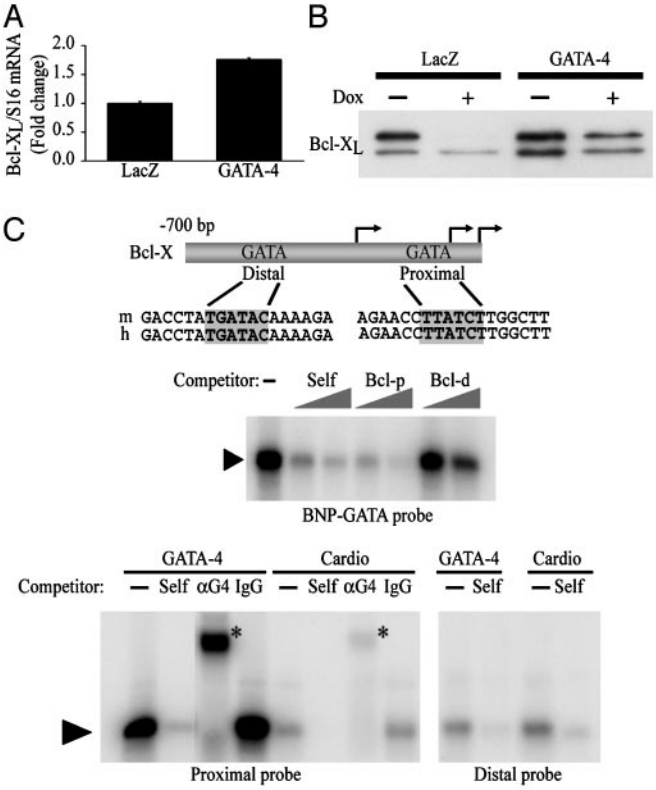


Fig. 5. Bcl-X is a GATA-4 target. (*A* and *B*) Quantitative PCR (*A*) and Western blots (*B*) were used to measure the level of Bcl-X_L mRNA and protein in cardiomyocytes infected with adenoviruses expressing LacZ or GATA-4. The data in *A* are the mean ± SEM of four independent experiments, and the results in *B* are representative of two independent experiments. (*C Top*) Schematic representation of the proximal promoter of the *Bcl-X* gene showing the two conserved GATA elements. (*C Middle*) Gel-shift analyses using nuclear extracts prepared from NIH 3T3-overexpressing GATA-4 or cardiomyocytes (Cardio) and the BNP-GATA, Bcl-X proximal-GATA (Bcl-p), and Bcl-X distal-GATA (Bcl-d) probes. (*C Bottom*) Supershift (indicated by asterisks) with the GATA-4 (αG4) antibody was used to confirm the identity of the DNA-binding complex in cardiomyocytes. Cold competitor DNAs were used at 25- and 100-fold excess. Arrowheads indicate GATA-4 binding. (*D*) The Bcl-X luciferase reporter was cotransfected with increasing amounts of GATA-4 expression vector in NIH 3T3 cells. (*E*) Bcl-X promoter activity in cardiomyocytes is decreased 5-fold after Dox treatment. In *D* and *E*, the data represent the mean ± SEM of four independent determinations.

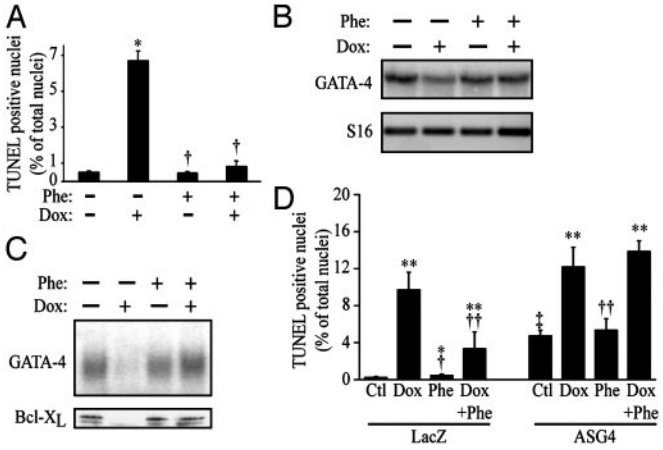


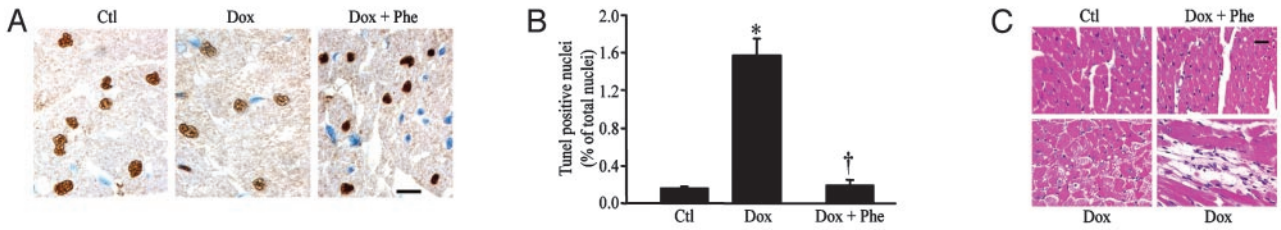
Fig. 6. GATA-4 is essential for α_1-adrenergic action. (*A*) Inhibition of Dox-induced apoptosis of cardiomyocytes by cotreatment with Phe for 12 h as determined by a TUNEL assay. The data are the mean ± SEM, (three to six per group; *, $P < 0.01$ vs. control; †, $P < 0.01$ vs. Dox). (*B* and *C*) Inhibition of Dox-induced change in gene expression. (*B*) Northern blot analyses after 12 h of Dox treatment in the presence or absence of Phe. (*C Upper*) Gel-shift analyses using cardiomyocyte nuclear extracts. (*C Lower*) Western blot showing Bcl-X_L protein in the same extracts. (*D*) Quantification of TUNEL assays in cardiomyocytes infected with adenovirus expressing LacZ or antisense GATA-4 transcripts (ASG4); the protective effect of Phe is abrogated in cells lacking GATA-4. The data are the mean ± SEM of two independent experiments carried out in duplicate [*, $P < 0.05$, and **, $P < 0.01$ vs. respective control (Ctl); †, $P < 0.05$, and ††, $P < 0.01$ vs. respective Dox; and ‡, $P < 0.01$ vs. control LacZ].

and most of them, such as bone morphogenetic proteins and retinoic acid, were characterized during embryonic development (36, 37). Previously, we showed that α1-adrenergic agonists enhance GATA-4 activity in postnatal cardiomyocytes, in part through phosphorylation of its activation domain (20); importantly, GATA-4 was essential for the cytoskeletal response of cardiomyocytes, arguing that GATA-4 was a downstream target as well as an effector of α1-agonists in the heart. Interestingly, α1-adrenoreceptor activation has been suggested to play an important role in cardioprotective mechanisms of ischemic preconditioning (38). We therefore used the α1-adrenergic agonist Phe to test whether manipulating GATA-4 activity might attenuate Dox cardiotoxicity and thus to establish the proof of principle that GATA-4 might be an accessible therapeutic target. Phe efficiently protected cardiomyocytes from Dox-induced apoptosis (Fig. 6*A*) and prevented Dox-induced depletion of GATA-4 and Bcl-X_L (Fig. 6 *B* and *C*). The antiapoptotic action of Phe depended on the presence of GATA-4 (Fig. 6*D*).

Next we tested the *in vivo* benefit of pharmacologic interference with GATA-4 depletion. Three groups of mice were analyzed: a control group, a group treated with a single dose of Dox, and a group cotreated with Phe and Dox. Low-dose Phe prevented GATA-4 depletion (Fig. 7*A*) and cardiomyocyte apoptosis (Fig. 7*B*). Moreover, in animals cotreated with Phe, myofibril structure appeared more preserved and there was no evidence of myocyte hypertrophy (Fig. 7*C*). Echocardiographic assessment revealed that, although 50% of animals treated with Dox developed CD, all animals in the control and Phe-treated groups maintained normal cardiac function (Fig. 7*D*). Left ventricular weight and heart rate were not altered by any treatment. Finally, unlike in the Dox-treated group, all animals receiving Phe and Dox were alive by the end of the 2-week protocol. We next tested the longer-term benefits of Phe; animals received a single injection of Dox in the presence or not of a 1-week infusion of Phe and monitored for up to 28 days (Fig. 7*E*); this short-term Phe treatment was protective against the Dox-induced CD that was manifested at later (28 days) time points. Together these results reveal that GATA-4 is an immediate early target of Dox action and that genetic or pharmacologic manipulation of GATA-4 activity can prevent Dox-induced cardiotoxicity.

Discussion

Previously we showed that depletion of GATA-4 interferes with *in vitro* cardiogenesis by down-regulating expression of cardioregulators and causing apoptosis of differentiating cardioblasts, whereas overexpression of GATA-4 up-regulated early



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	FS (%)		CD		SBP (mmHg)		Survival (alive/total)
Days of follow-up	7	14	7	14	7	14	14
Ctl	56.0 ± 1.4	60.7 ± 0.9	0/14	0/6	112.0 ± 3.7	111.4 ± 3.5	6/6
Dox	48.0 ± 1.9*	42.6**	10/22*	1/1	130.2 ± 2.1**	N.D.	1/6*
Dox + Phe	59.8 ± 1.4††	58.9 ± 0.5††	0/10†	0/4	116.9 ± 3.2†	126.4 ± 1.3*	4/4†

E

	HR (beats/min)		FS (%)		CD	
Days of follow-up	14	28	14	28	14	28
Ctl	741 ± 11	751 ± 4	59.1 ± 2.3	61.1 ± 1.6	0/8	0/8
Dox	746 ± 13	734 ± 4	60.1 ± 3.5	52.9 ± 4.1*	0/4	2/4*
Dox + Phe	722 ± 24	709 ± 45	61.0 ± 1.6	68.9 ± 1.2†	0/3	0/3

Fig. 7. Prevention of Dox cardiotoxicity by infusion of α1-adrenergic agonist *in vivo*. (*A*) GATA-4 protein levels (brown nuclei) were determined in the ventricles of the control (Ctl) and Dox- and Dox+Phe-treated mice by using immunohistochemistry. (Scale bar, 10 μm.) (*B*) Quantification of TUNEL assay in the ventricles of control ($n = 3$) and Dox- ($n = 4$) and Dox+Phe- ($n = 4$) treated mice. The data represent mean ± SEM (*, $P < 0.05$ vs. control; †, $P < 0.05$ vs. Dox). (*C*) Left ventricle tissue sections of control and Dox- and Dox+Phe-treated mice stained with hematoxylin and eosin. (Scale bar, 20 μm.) (*D*) Phe prevention of Dox cardiotoxicity. FS, frequency of CD, systolic blood pressure (SBP), and survival were determined in control and Dox- and Dox + Phe-treated mice. Data are mean ± SEM or frequency ratios of three separate experiments, with $n = 8$–22 in the 1-week follow-up group and $n = 1$–6 in the 2-week follow-up group (*, $P < 0.05$, and **, $P < 0.01$ vs. control; †, $P < 0.05$, and ††, $P < 0.01$ vs. Dox). (*E*) Phe prevention of chronic Dox cardiotoxicity. Heart rate (HR), FS, and frequency of CD were determined in control and Dox- and Dox+Phe-treated mice. Data are mean ± SEM or frequency ratios, with $n = 3$–8 (*, $P < 0.05$ vs. control; †, $P < 0.01$ vs. Dox).

differentiation markers and enhanced cardiogenesis (18). The data presented extend the role of GATA-4 as a survival factor to terminally differentiated myocytes and reveals that the embryonic and postnatal hearts share similar regulators. Interestingly, although a 50% reduction in GATA-4 levels did not interfere with normal embryonic or postnatal mouse development (24), it did impair the drug-induced stress response, raising the possibility that GATA-4 may be an essential component of the adaptive response of the adult heart. This would be consistent with previous findings that GATA elements are essential to mediate *in vivo* transcriptional response to pressure or volume overload (39–41) as well as our own *in vitro* findings that GATA-4 is essential for cytoskeletal reorganization of cardiomyocytes (20).

Dox-induced depletion of GATA-4 may well explain the maximal cumulative dose tolerance and the latent cardiomyopathy observed in patients treated with anthracyclines. Support for this hypothesis comes from the finding that mice with 50% reduction in GATA-4 levels are hyperresponsive to Dox and display greater myocyte loss and CD than wild-type littermates. Moreover, cardiomyocytes with reduced GATA-4 levels are unresponsive to several stimuli (20) and have reduced expression of several contractile genes and genes encoding hormones, receptors, and ion channels (ref. 19; unpublished data). Thus, a decrease in GATA-4 levels in the heart of patients treated with Dox ultimately would impair their ability to respond to work overload and other stimuli that produce cardiac stress. Restoring or preventing GATA-4 depletion may be an effective approach to cardioprotection.

In this respect, regulation of GATA-4 levels by α1-adrenergic agonists is noteworthy, because they are in clinical use already (42). The exact mechanism(s) by which α1-adrenergic stimulation counteracts Dox cardiotoxicity is not fully understood yet, but two points are noteworthy. First, although activation of α1-adrenoreceptors can trigger numerous intracellular signaling cascades, their antiapoptotic effect required GATA-4 and was abrogated by antisense-mediated GATA-4 down-regulation (Fig. 6*D*). Second, this protective effect was dissociated from the hypertrophic effect of Phe; in culture, the effect of Phe on GATA-4 and apoptosis was observed after only 6 h of treatment in the absence of any biochemical (ANF) or morphologic evidence of myocyte hypertrophy. Finally, *in vivo*, the low dose of Phe used did not produce any myocyte hypertrophy or increase in heart weight (Fig. 7). Still, by preventing depletion of GATA-4, α1-agonists maintain cardiac gene expression and myofibril integrity as well as the ability of myocytes to respond to extracellular stimuli and work overload. The use of these agents in combination therapy with Dox may be beneficial to patients undergoing chemotherapy. Other small molecules that stabilize or enhance GATA-4 levels may be cardioprotective also.

The essential role of GATA-4 as a survival factor may be explained, at least in part, by its function as an upstream activator of *Bcl-X* and hence mitochondrial function and integrity. Recent work using adenoviral-mediated overexpression of *Bcl-*X_L revealed a protective effect against Dox-induced apoptosis and ischemic injury (43). The finding that GATA-4 is essential for survival of terminally differentiated, growth-arrested myocytes and that it directly regulates transcription of the antiapoptotic gene *Bcl-*X_L provides mechanistic insight for the role of GATA and other tissue-specific transcription factors in cell survival independent of differentiation and proliferation. GATA factors are DNA-binding proteins that play essential roles in lineage differentiation and organogenesis (44, 45). Gene-inactivation studies have suggested a link between GATA factors and cell survival, but it was always associated with differentiation or cell-cycle progression (46–48). Overexpression of GATA-1 in differentiating erythroid cells was also shown to up-regulate Bcl-2 and *Bcl-*X_L, although it was again associated with differentiation (49, 50). More recently, Kim *et al.* (51) showed a correlation between GATA-4 expression and apoptosis in an immortalized atrial cell line, but whether this occurred indepen-

dent of proliferation and/or differentiation was not discussed. Thus, the data presented in this article show that GATA factors directly regulate the apoptosis program in postnatal, terminally differentiated cells by transcriptional activation of *Bcl-X*. Modulation of survival/apoptosis genes by tissue-specific transcription factors may be a general paradigm that can be exploited effectively for cell-specific regulation of apoptosis in disease states.

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1. Olson, E. N. & Schneider, M. D. (2003) *Genes Dev*. **17,** 1937–1956.
2. Regula, K. M., Ens, K. & Kirshenbaum, L. A. (2003) *J. Mol. Cell Cardiol*. **35,** 559–567.
3. Yamamoto, S., Yang, G., Zablocki, D., Liu, J., Hong, C., Kim, S. J., Soler, S., Odashima, M., Thaisz, J., Yehia, G., *et al*. (2003) *J. Clin. Invest*. **111,** 1463–1474.
4. Wencker, D., Chandra, M., Nguyen, K., Miao, W., Garantziotis, S., Factor, S. M., Shirani, J., Armstrong, R. C. & Kitsis, R. N. (2003) *J. Clin. Invest*. **111,** 1497–1504.
5. Shi, Y. (2002) *Mol. Cell* **9,** 459–470.
6. Ashkenazi, A. (2002) *Nat. Rev. Cancer* **2,** 420–430.
7. Strasser, A., O'Connor, L. & Dixit, V. M. (2000) *Annu. Rev. Biochem*. **69,** 217–245.
8. Adams, J. M. (2003) *Genes Dev*. **17,** 2481–2495.
9. Cory, S. & Adams, J. M. (2002) *Nat. Rev. Cancer* **2,** 647–656.
10. Olivetti, G., Abbi, R., Quaini, F., Kajstura, J., Cheng, W., Nitahara, J. A., Quaini, E., Di Loreto, C., Beltrami, C. A., Krajewski, S., *et al*. (1997) *N. Engl. J. Med*. **336,** 1131–1141.
11. Chen, Z., Chua, C. C., Ho, Y. S., Hamdy, R. C. & Chua, B. H. (2001) *Am. J. Physiol*. **280,** H2313–H2320.
12. Singal, P. K. & Iliskovic, N. (1998) *N. Engl. J. Med*. **339,** 900–905.
13. Lipshultz, S. E., Lipsitz, S. R., Mone, S. M., Goorin, A. M., Sallan, S. E., Sanders, S. P., Orav, E. J., Gelber, R. D. & Colan, S. D. (1995) *N. Engl. J. Med*. **332,** 1738–1743.
14. Ito, H., Miller, S. C., Billingham, M. E., Akimoto, H., Torti, S. V., Wade, R., Gahlmann, R., Lyons, G., Kedes, L. & Torti, F. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4275–4279.
15. Jeyaseelan, R., Poizat, C., Baker, R. K., Abdishoo, S., Isterabadi, L. B., Lyons, G. E. & Kedes, L. (1997) *J. Biol. Chem*. **272,** 22800–22808.
16. Jeyaseelan, R., Poizat, C., Wu, H. Y. & Kedes, L. (1997) *J. Biol. Chem*. **272,** 5828–5832.
17. Poizat, C., Sartorelli, V., Chung, G., Kloner, R. A. & Kedes, L. (2000) *Mol. Cell. Biol*. **20,** 8643–8654.
18. Grépin, C., Nemer, G. & Nemer, M. (1997) *Development (Cambridge, U.K.)* **124,** 2387–2395.
19. Charron, F., Paradis, P., Bronchain, O., Nemer, G. & Nemer, M. (1999) *Mol. Cell. Biol*. **19,** 4355–4365.
20. Charron, F., Tsimiklis, G., Arcand, M., Robitaille, L., Liang, Q., Molkentin, J. D., Meloche, S. & Nemer, M. (2001) *Genes Dev*. **15,** 2702–2719.
21. Pehlivan, T., Pober, B. R., Brueckner, M., Garrett, S., Slaugh, R., Van Rheeden, R., Wilson, D. B., Watson, M. S. & Hing, A. V. (1999) *Am. J. Med. Genet*. **83,** 201–206.
22. Morin, S., Charron, F., Robitaille, L. & Nemer, M. (2000) *EMBO J*. **19,** 2046–2055.
23. Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C. & Lodish, H. F. (1999) *Cell* **98,** 181–191.
24. Molkentin, J. D., Lin, Q., Duncan, S. A. & Olson, E. N. (1997) *Genes Dev*. **11,** 1061–1072.
25. Paradis, P., Dali-Youcef, N., Paradis, F. W., Thibault, G. & Nemer, M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 931–936.
26. Yang, X. P., Liu, Y. H., Rhaleb, N. E., Kurihara, N., Kim, H. E. & Carretero, O. A. (1999) *Am. J. Physiol*. **277,** H1967–H1974.
27. Nemer, G. & Nemer, M. (2002) *Development (Cambridge, U.K.)* **129,** 4045–4055.
28. Levin, E. R., Gardner, D. G. & Samson, W. K. (1998) *N. Engl. J. Med*. **339,** 321–328.
29. McBride, K. & Nemer, M. (2001) *Can. J. Physiol. Pharmacol*. **79,** 673–681.
30. Morin, S., Paradis, P., Aries, A. & Nemer, M. (2001) *Mol. Cell. Biol*. **21,** 1036–1044.
31. McBride, K. & Nemer, M. (1998) *Mol. Cell. Biol*. **18,** 5073–5081.
32. Argentin, S., Ardati, A., Tremblay, S., Lihrmann, I., Robitaille, L., Drouin, J. & Nemer, M. (1994) *Mol. Cell. Biol*. **14,** 777–790.
33. Morimoto, T., Hasegawa, K., Wada, H., Kakita, T., Kaburagi, S., Yanazume, T. & Sasayama, S. (2001) *J. Biol. Chem*. **276,** 34983–34989.
34. Hautala, N., Tokola, H., Luodonpaa, M., Puhakka, J., Romppanen, H., Vuolteenaho, O. & Ruskoaho, H. (2001) *Circulation* **103,** 730–735.
35. Grillot, D. A., Gonzalez-Garcia, M., Ekhterae, D., Duan, L., Inohara, N., Ohta, S., Seldin, M. F. & Nunez, G. (1997) *J. Immunol*. **158,** 4750–4757.
36. Schultheiss, T. M., Burch, J. B. & Lassar, A. B. (1997) *Genes Dev*. **11,** 451–462.
37. Ghatpande, S., Ghatpande, A., Zile, M. & Evans, T. (2000) *Dev. Biol*. **219,** 59–70.
38. Salvi, S. (2001) *Chest* **119,** 1242–1249.
39. Marttila, M., Hautala, N., Paradis, P., Toth, M., Vuolteenaho, O., Nemer, M. & Ruskoaho, H. (2001) *Endocrinology* **142,** 4693–4700.
40. Hasegawa, K., Lee, S. J., Jobe, S. M., Markham, B. E. & Kitsis, R. N. (1997) *Circulation* **96,** 3943–3953.
41. Herzig, T. C., Jobe, S. M., Aoki, H., Molkentin, J. D., Cowley, A. W., Jr., Izumo, S. & Markham, B. E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 7543–7548.
42. Stewart, J. M., Munoz, J. & Weldon, A. (2002) *Circulation* **106,** 2946–2954.
43. Huang, J., Ito, Y., Morikawa, M., Uchida, H., Kobune, M., Sasaki, K., Abe, T. & Hamada, H. (2003) *Biochem. Biophys. Res. Commun*. **311,** 64–70.
44. Cantor, A. B. & Orkin, S. H. (2002) *Oncogene* **21,** 3368–3376.
45. Patient, R. K. & McGhee, J. D. (2002) *Curr. Opin. Genet. Dev*. **12,** 416–422.
46. Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. & Grosveld, F. (1999) *Development (Cambridge, U.K.)* **126,** 723–732.
47. Weiss, M. J. & Orkin, S. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9623–9627.
48. Pandolfi, P. P., Roth, M. E., Karis, A., Leonard, M. W., Dzierzak, E., Grosveld, F. G., Engel, J. D. & Lindenbaum, M. H. (1995) *Nat. Genet*. **11,** 40–44.
49. Gregory, T., Yu, C., Ma, A., Orkin, S. H., Blobel, G. A. & Weiss, M. J. (1999) *Blood* **94,** 87–96.
50. Tanaka, H., Matsumura, I., Nakajima, K., Daino, H., Sonoyama, J., Yoshida, H., Oritani, K., Machii, T., Yamamoto, M., Hirano, T., *et al*. (2000) *Blood* **95,** 1264–1273.
51. Kim, Y., Ma, A. G., Kitta, K., Fitch, S. N., Ikeda, T., Ihara, Y., Simon, A. R., Evans, T. & Suzuki, Y. J. (2003) *Mol. Pharmacol*. **63,** 368–377.