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Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells *in vivo* and *in vitro*

(adriamycin/anthracycline/cardiotoxicity/cardiomyopathy/sarcomere)

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ABSTRACT **The anthracycline antibiotic doxorubicin produces a characteristic myopathy in cardiac muscle that limits its use in cancer therapy. We have shown in cultured neonatal rat cardiac muscle cells that doxorubicin treatment resulted in a rapid, selective decrease in the expression of muscle-specific genes, which preceded other changes characteristic of doxorubicin cardiomyopathy. Doxorubicin selectively and dramatically decreased the levels of mRNA for the sarcomeric genes, α-actin, troponin I, and myosin light chain 2, as well as the muscle-specific, but nonsarcomeric M isoform of creatine kinase. However, doxorubicin did not affect nonmuscle gene transcripts (pyruvate kinase, ferritin heavy chain, and β-actin). Actinomycin D, an inhibitor of DNA-dependent RNA polymerase, did not show a similar selective decrease of muscle-specific mRNAs but, rather, produced a nonspecific, dose-dependent decrease of muscle and nonmuscle transcripts. The doxorubicin effect on muscle gene expression was limited to cardiac muscle; cultured skeletal myocytes were resistant to the effects of doxorubicin at 100-fold greater doses than those causing changes in mRNA levels in cardiac muscle cells. These effects of doxorubicin were reproduced *in vivo*; rats injected with doxorubicin showed a dose-dependent decrease in the levels of mRNAs for α-actin, troponin I, myosin light chain 2, and M isoform of creatine kinase in cardiac but not skeletal muscle. These selective changes in gene expression in cardiocyte cultures and cardiac muscle precede classical ultrastructural changes and may explain the myofibrillar loss that characterizes doxorubicin cardiac injury.**

Doxorubicin is one of the most effective and widely used chemotherapeutic agents. It is used in the treatment of leukemias, lymphomas, and various solid tumors. However, this drug has cardiotoxic effects that restrict its full clinical potential. As cumulative drug doses exceed 500 mg/m^2, the incidence of congestive heart failure increases rapidly, occurring in 15–30% of patients who have exceeded this dose. More than half of these patients die as a direct result of heart failure (1). Great effort has been directed at dissociating the beneficial effects of this drug from its cardiotoxicity by modifying the schedule of administration of doxorubicin and through evaluation of the ultrastructural changes in heart muscle (2, 3). However, the mechanism underlying cardiotoxicity remains uncertain. Empiric attempts to limit cardiac toxicity have thus far only been partially successful [see review (4)].

In myocardial tissue isolated by endomyocardial biopsy from humans treated with doxorubicin, the primary ultrastructural change is disruption and loss of myofibrils (5). Alterations of the Z-band structure and disarray of the thin filaments are observed with doxorubicin treatment *in vivo* (6) and *in vitro* (7, 8). The mechanism responsible for these changes is not understood.

In this study we have addressed the hypothesis that the structural disintegration of myofibrils observed in doxorubicin treatment might be linked to alterations in the expression of genes coding for sarcomeric proteins.

MATERIALS AND METHODS

Cells and Cell Culture. Primary cultures of neonatal rat cardiac muscle cells were prepared by the method described by Simpson and Savion (9) and incubated for 48 hr before treatment with doxorubicin or actinomycin D. Differentiated human skeletal muscle cells were prepared from primary cultures of human skeletal myoblasts (10) as described (11). In certain experiments, 10 μM cytosine arabinonucleoside was added to remove replicating cells (11). The rat skeletal muscle cell line L8 was cultured in Dulbecco's modified Eagle medium (DMEM) plus 20% fetal calf serum. Differentiation was initiated by replacing the medium with DMEM plus 2% horse serum; cells were maintained in this medium for 6 days before treatment with doxorubicin.

[^{3}H]Leucine Incorporation. The doxorubicin effect on protein synthesis in cultured cardiac cells was evaluated by trichloroacetic acid precipitation following incubation with 1 μCi of [^{3}H]leucine (1 Ci = 37 GBq) for 4 hr.

Sample Preparation for Electron Microscopy. Cultured cardiac muscle cells and rat heart tissue were fixed for 2 hr in 1% glutaraldehyde in 0.067 M sodium cacodylate (pH 7.4), washed in buffer, and postfixed in 2% osmium tetroxide for 1 hr. The samples were prestained in 1% uranyl acetate, dehydrated in a graded ethanol series, and embedded in 100% epoxy resin. After sectioning, samples were mounted on parlodian-coated grids, stained with lead citrate, and examined with a Philips 201C electron microscope.

Immunofluorescence Staining and Cell Count. Cardiac muscle cells were distinguished from nonmuscle cells by immunofluorescence staining of myosin heavy chain (MHC) as described by Silberstein *et al.* (12). The monoclonal antibody for myosin heavy chain (4A.1025) was a gift of H. Blau (Stanford, CA). Phase-contrast and fluorescence fields were

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Abbreviations: MLC, myosin light chain; PK, pyruvate kinase; FH, ferritin heavy chain; M-CK, M isoform of creatine kinase; MHC, myosin heavy chain.

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projected on a screen and cell numbers from 10 random fields were counted.

Isolation and Analysis of RNA. RNA was isolated from cells or tissue by lysis in guanidine thiocyanate and centrifugation through a 5.7 M CsCl cushion (13). RNA was analyzed by Northern or dot blotting followed by hybridization to [^{32}P]cDNA probes prepared by the random primer method (14) as described (11, 15).

cDNA Probes. The following cDNAs were used in this study: human cardiac α-actin (16), human β-actin (17), rat cardiac troponin I (R.W. and R.G., unpublished data), human myosin light chain 2 (MLC 2) slow (G.L. and R.W., unpublished data), rat pyruvate kinase (PK) (18), mouse ferritin heavy chain (FH) (15), and human M isoform of creatine kinase (M-CK) (19).

***In Vivo* Experiments.** Male Sprague–Dawley rats (150–200 g) were anesthetized by intraperitoneal injection of 10 mg of xylazine per kg and 50 mg of ketamine hydrochloride per kg and injected intravenously with varying doses of doxorubicin (dissolved in normal saline). Control rats received an injection of normal saline. Twenty-four hours after the injection rats were sacrificed by asphyxiation. For the dose–response experiments, RNAs from five rat hearts per each dose group (2, 20, and 40 mg/kg) and six controls were evaluated by dot blots. In separate experiments designed to compare gene expression in cardiac and skeletal muscle, femoral muscle and heart were removed from the same animals (three control rats and four rats treated with doxorubicin at 20 mg/kg). The RNA was analyzed by Northern blots. Hearts obtained from control and doxorubicin-treated (20 mg/kg) animals were also processed for electron microscopy.

Statistical Analyses. One-way analysis of variance and a multiple comparison procedure (Scheffe *F* test) were used for statistical analyses.

RESULTS

Ultrastructural Changes and Effects on Cell Density. Cultured neonatal rat cardiac muscle cells treated for 48 hr with 1 μM doxorubicin showed changes characteristic of doxorubicin toxicity, myofibrillar loss and sarcotubular swelling, as well as nonspecific changes—i.e., mitochondrial damage (Fig. 1 *Upper*). To select a dose and duration of doxorubicin treatment that would model early stages of cardiotoxicity, we examined the effect of lower concentrations and more abbreviated treatment of doxorubicin on cardiac muscle cell structure. After 24 hr with 0.5 μM doxorubicin, cells showed little ultrastructural change except for the loss of cytoplasmic projections. No change in myofilaments, mitochondria, or sarcoplasmic reticulum was observed at this time point (Fig. 1 *Lower*, a and b). In cells treated for 24 hr with 0.5 μM doxorubicin, there was no effect on cell density or morphology when compared with control cultures (Fig. 1 *Lower*, c and d). The percentage of cardiac muscle cells in the culture was examined by staining for MHC expression using immunofluorescence microscopy. The percentage of cardiac muscle cells was unchanged up to 24 hr of 0.5 μM doxorubicin treatment—88.8% ± 4.6% in control and 89.7% ± 3.0% in doxorubicin-treated cells (Fig. 1 *Lower*, e and f). Thus, although a longer exposure of doxorubicin at 1 μM produced classic cardiac injury, as shown in Fig. 1 *Lower*, an exposure for 24 hr at 0.5 μM had minimal morphological and ultrastructural effects.

Doxorubicin Effect on Protein Synthesis. To further explore the dose and schedule dependency of doxorubicin on cardiac muscle cells, we examined various concentrations of doxorubicin for an effect on protein synthesis using [^{3}H]leucine incorporation. There was no effect on protein synthesis after 8 hr of treatment with 0.2 μM doxorubicin. With 0.5 μM doxorubicin, there was a slight (25%) inhibition at 8 hr. After

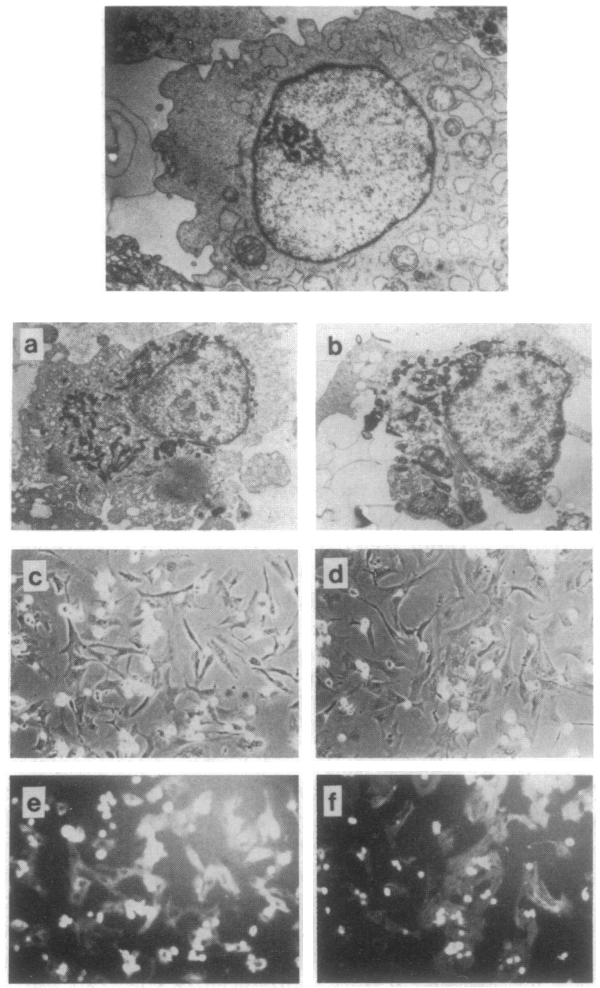

FIG. 1. (*Upper*) Ultrastructural changes observed in cultured neonatal rat cardiac muscle cell treated with 1 μM doxorubicin for 48 hr. The characteristic changes of doxorubicin toxicity, such as myofibrillar loss and sarcotubular swelling, as well as nonspecific changes, are observed. (×6700.) Compare with control (a, *Lower*). (*Lower*) Control (a, c, and e) and doxorubicin-treated (0.5 μM, 24 hr) (b, d, and f) neonatal rat cardiac muscle cells in culture as observed by electron microscopy (a and b) (×4300), phase-contrast microscopy (c and d), and immunofluorescence staining for MHC (e and f). No difference was observed between control and doxorubicin-treated cells with any of these techniques. Note that the "ground-glass" appearance of the specific MHC staining (e and f) was not altered under these experimental conditions.

24 hr of treatment with 0.2 and 0.5 μM doxorubicin, protein synthesis was inhibited by 41% and 68%, respectively (data from duplicate dishes).

Doxorubicin Effect on Gene Expression. Based on the results above, 0.2 and 0.5 μM concentrations of doxorubicin were chosen to examine effects on gene expression. As seen in Fig. 2 *Left* (similar results were obtained in four separate experiments), there was a dose- and time-dependent decrease in muscle-specific mRNAs but not in β-actin (cytoskeletal actin), FH, or PK mRNAs. Quantitation of the Northern blots shown in Fig. 2 *Left* by densitometry revealed that at 24 hr α-actin (sarcomeric actin) mRNA decreased to 20% of control expression with 0.2 μM doxorubicin treatment and was nearly undetectable (<10% of control levels) with 0.5 μM doxorubicin. The amount of troponin I and MLC 2 mRNA was also markedly reduced at 24 hr. For MLC 2, levels fell to 12% of control with 0.2 μM and to 4% of control with 0.5 μM doxorubicin treatment. Similarly, troponin I mRNA decreased to 10% of control levels at the 0.2 μM dose

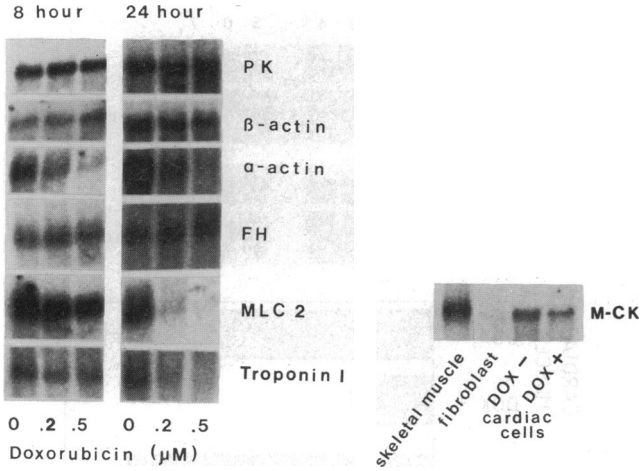

FIG. 2. (*Left*) Northern blots of RNA from doxorubicin-treated neonatal rat cardiac cell cultures hybridized to radiolabeled DNA specific for the transcripts identified on the right. (*Right*) Northern blots of RNA from doxorubicin-treated neonatal rat cardiac muscle cells probed with M-CK. Cardiac muscle cells were treated for 24 hr with 0.5 μM doxorubicin (DOX). RNAs from human skeletal muscle cells and rat cardiac fibroblasts were also analyzed in order to establish the specificity for the M-CK.

and was undetectable at 0.5 μM. Further, these decreases in muscle-specific mRNAs with 0.2 μM doxorubicin were observed after only 8 hr, a time point preceding any effect on total protein synthesis. For example, at 8 hr 0.2 μM doxorubicin caused a 49%, 62%, and 54% reduction of α-actin, MLC 2, and troponin I transcripts, respectively.

To determine whether the effect of doxorubicin was limited to sarcomeric genes, we examined whether doxorubicin treatment would lead to a decrease in the mRNA for a nonsarcomeric, but muscle-specific protein, such as the M-CK (19, 20). Treatment of cultured cardiac muscle cells for 24 hr with 0.5 μM doxorubicin led to a marked reduction of the muscle-specific M-CK mRNA (Fig. 2 *Right*).

Do Nonmuscle Cells Contribute to the Doxorubicin Effect on Gene Expression? Our cardiac cultures contain ≈10% cells negative for MHC by immunofluorescence ("nonmuscle cells"). To examine whether the apparent selective effect of doxorubicin on muscle gene expression merely reflected the relative resistance of these nonmuscle cells to doxorubicin (that nonmuscle cells made a disproportionate contribution to the total mRNA for β-actin, FH, and PK), we further reduced the proportion of nonmuscle cells with cytosine arabinonucleoside treatment. After 48 hr of pretreatment with 10 μM cytosine arabinonucleoside, cells were treated with and without 0.5 μM doxorubicin for 24 hr in the presence of cytosine arabinonucleoside. At the end of the cytosine arabinonucleoside treatment, the percentage of MHC stained cells as determined by immunofluorescence microscopy was 98%, as compared to 89% in the absence of cytosine arabinonucleoside. The effect of doxorubicin on β-actin, FH, and PK transcripts was identical in cytosine arabinonucleoside-treated cells and control cells (data not shown), demonstrating that nonmuscle cells did not contribute to the selective decrease in muscle-specific transcripts observed with doxorubicin.

Actinomycin D Effect on Gene Expression in Cardiac Cells. We also considered the possibility that the selectivity of doxorubicin on muscle-specific mRNAs might in fact result from a nonspecific inhibition of gene expression—that this apparently selective effect might be a consequence of shorter half-lives for muscle-specific transcripts. To test this possibility, we used actinomycin D, an inhibitor of DNA-dependent RNA polymerase. Actinomycin D did not show a selective inhibition of muscle-specific transcripts similar to doxorubicin but, rather, produced a nonspecific, dose-dependent inhibition of muscle and nonmuscle mRNAs (Fig. 3).

Doxorubicin Effect on Gene Expression in Skeletal Muscle Cells. Generally, it is thought that skeletal muscle is more resistant to doxorubicin toxicity than cardiac muscle. Is the selective decrease in muscle-specific gene expression by doxorubicin limited to cardiac cells? To approach this question, we performed Northern blot analysis with RNA isolated from doxorubicin-treated cultures of primary human skeletal muscle and rat myocytes. Cultured skeletal myotubes (see *Materials and Methods*) were resistant to any effect of doxorubicin on the mRNAs examined. Even 50 μM doxorubicin treatment for 24 hr (100 times the concentration at which changes in gene expression were observed in cardiac muscle cells) caused no decrease of either muscle-specific (α-actin, M-CK) or nonmuscle (PK) transcripts in skeletal muscle cells (Fig. 4). An experiment of identical design was performed using rat skeletal muscle L8 cells. Differentiated L8 cells were also resistant up to 50 μM doxorubicin treatment (data not shown).

Doxorubicin Effect on Gene Expression and Ultrastructure in Adult Rat Hearts. To determine whether doxorubicin effects on muscle-specific mRNA were limited to cardiac muscle cells in tissue culture, we performed an *in vivo* study using adult rats. Total RNA was isolated from the hearts of control rats and rats treated with doxorubicin (doses of 2, 20, and 40 mg/kg) for 24 hr. Dot blots were probed with α-actin, troponin I, MLC 2, and M-CK cDNA. Autoradiograms were analyzed by densitometry, and the results were normalized for PK expression. A dose-dependent reduction in muscle-specific mRNA was observed (Fig. 5).

To compare the doxorubicin effect on cardiac and skeletal muscle *in vivo*, cardiac and skeletal mRNAs from three control and four doxorubicin-treated rats were analyzed by Northern blots. Neither α-actin (Fig. 6) nor the M-CK (data not shown) mRNA levels were changed in skeletal muscle,

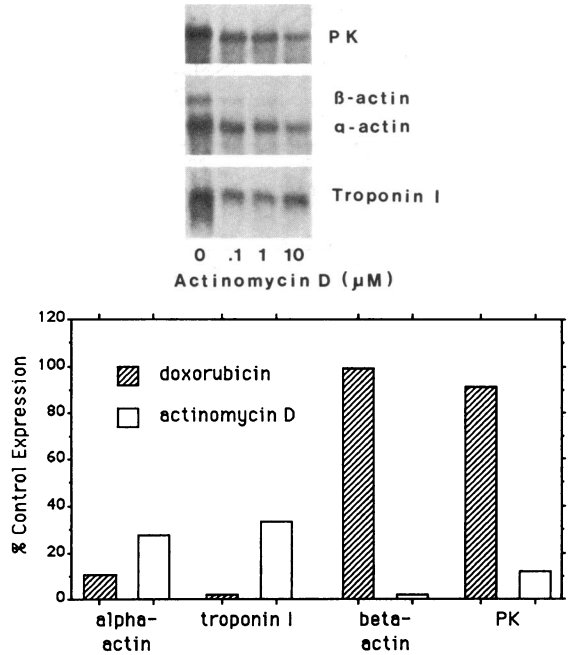

FIG. 3. (*Upper*) Northern blots of RNA from rat cardiac muscle cells in culture treated with actinomycin D for 24 hr and hybridized with radiolabeled DNA probes identified on the right. (*Lower*) Results from doxorubicin- (0.5 μM, 24 hr) and actinomycin D- (10 μM, 24 hr) treated cultures were compared after normalizing for expression in nontreated cultures.

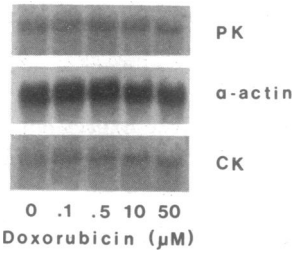

FIG. 4. Northern blots of RNA from doxorubicin-treated human myotubes in culture.

despite the inhibition of α-actin and M-CK gene expression in cardiac muscle isolated from the same rats.

Tissue samples from hearts of control and doxorubicin-treated animals (20 mg/kg) from these experiments were prepared for electron microscopy and examined for evidence of cardiotoxicity. In contrast to the marked effect on mRNA levels observed after 24 hr, no remarkable ultrastructural changes were observed up to 3 days after the injection of 20 mg of doxorubicin per kg. However, 8 days after the treatment, most of the myocardium showed myofibrillar loss and sarcotubular swelling as well as other evidence of cell injury, such as mitochondrial damage (Fig. 7).

DISCUSSION

Although the last 15 years have witnessed considerable effort toward clarifying the mechanism of doxorubicin cardiotoxicity, the sequence of events leading to myocardial damage has remained elusive. One widely held hypothesis associates doxorubicin cardiotoxicity with the generation of oxygen free radicals that disrupt DNA, membrane proteins, and phospholipids (21–23). Other hypotheses with strong experimental support have also been proposed, including a role for a metabolite of doxorubicin (24) in the etiology of cardiotoxicity. Several studies have suggested the interaction of doxorubicin with myofibrillar proteins in the etiology of doxorubicin cardiotoxicity (25, 26).

To explore the early events of doxorubicin cardiotoxicity, we used primary cardiac muscle cell cultures prepared from neonatal rats as a model system. Doses (0.2 μM and 0.5 μM) of doxorubicin that approximate peak plasma concentrations of doxorubicin after intravenous administration in humans

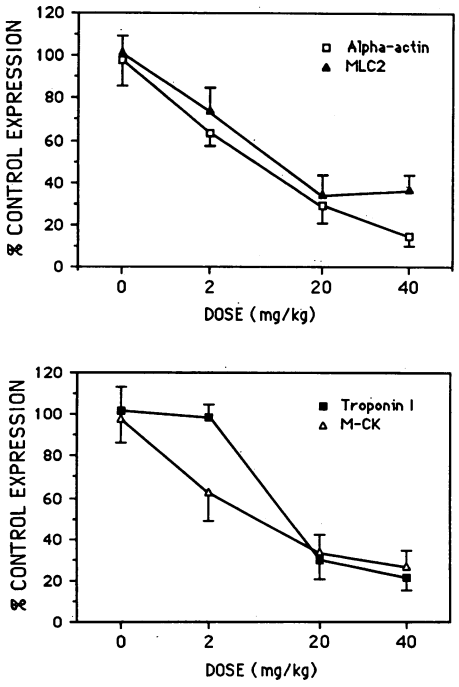

FIG. 5. Expression of muscle-specific genes in doxorubicin-treated hearts from adult rats. Total RNA from hearts isolated from rats treated with various doses of doxorubicin was applied to nitrocellulose in a dot blot apparatus. The autoradiograms were analyzed by densitometry and the results are expressed as the ratio of α-actin, troponin I, MLC 2, or M-CK normalized to PK. Data are shown as the mean ± SEM of five rats per each dose group and six rats in the control group. Significant differences from the control level of expression were observed for all muscle-specific gene expression at 20 mg/kg and 40 mg/kg ($P < 0.01$).

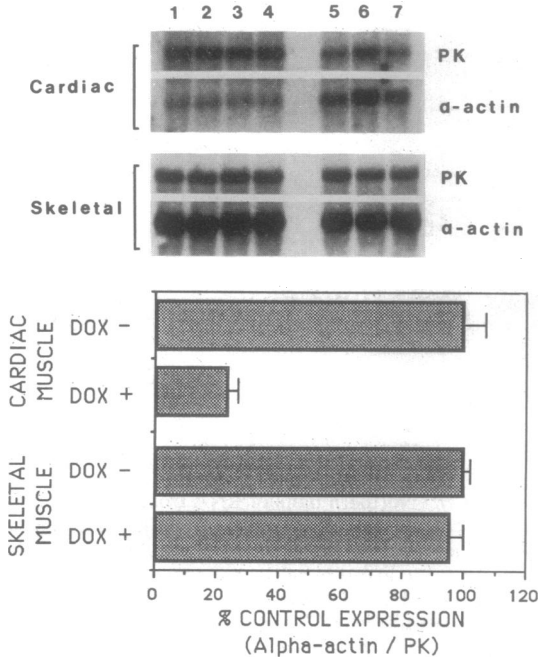

FIG. 6. (*Upper*) Northern blots of cardiac and skeletal muscle RNA from doxorubicin-treated (20 mg/kg for 24 hr) (lanes 1–4) and control (lanes 5–7) rats probed with α-actin. PK is used to normalize α-actin expression for variability in sample loading. (*Lower*) Autoradiograms of the Northern blot in *A* were analyzed by densitometry. The results of α-actin gene expression normalized to PK were compared between cardiac and skeletal muscle. α-Actin mRNA is not affected by doxorubicin (DOX) treatment despite significant ($P < 0.01$) inhibition in cardiac muscle.

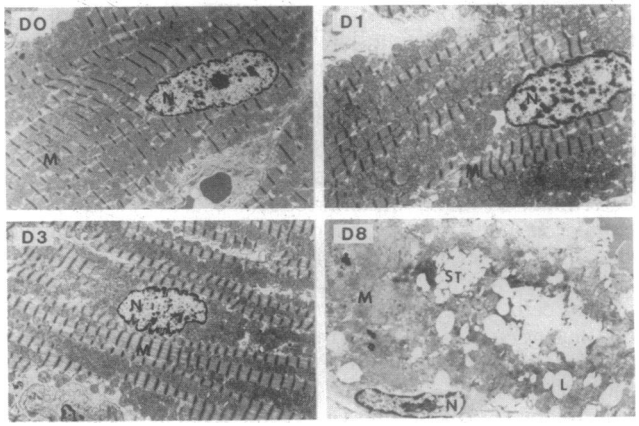

FIG. 7. Ultrastructural findings in adult rat heart tissue following doxorubicin treatment. Doxorubicin (20 mg/kg) was injected intravenously into the rats, and samples were taken 1 day (D1), 3 days (D3), and 8 days (D8) after the injection. A sample without treatment (D0) is presented as a control. (×2000.) N, myocyte nucleus; M, myofibrils; ST, sarcotubular swelling; L, intracellular lipid droplet (degenerating myocyte).

(27), and that model early stages of doxorubicin cardiotoxicity, were chosen. With these concentrations, the steady-state levels of the mRNAs of three sarcomeric muscle genes, α-actin, MLC 2 (slow), and cardiac troponin I, and one muscle-specific, but nonsarcomeric gene, the M-CK, were all markedly decreased by doxorubicin treatment (Fig. 2). In contrast, the transcripts of three nonmuscle genes were unaffected. Nonmuscle mRNAs were chosen from genes involved in different cellular functions: β-actin, in cytoskeletal structure; FH, in iron storage; and PK, in glycolysis. The spectrum of muscle genes affected suggests that doxorubicin toxicity is not limited to the sarcomere but involves cardiac cellular function more generally.

To directly examine whether the apparent selectivity of doxorubicin for muscle genes was due to shorter half-life of muscle mRNAs as compared to nonmuscle mRNAs, we compared the effects of actinomycin D and doxorubicin on cardiac muscle cells. Actinomycin D treatment of cardiac muscle cell cultures produced a nonspecific, dose-dependent decrease of muscle and nonmuscle gene transcripts, in contrast to the selective alteration of muscle gene expression seen with doxorubicin. This excludes the possibility that doxorubicin is acting at these doses as a nonspecific inhibitor of transcription in muscle cells, although the relative contribution of transcriptional and posttranscriptional changes to the selective effect of doxorubicin on muscle-specific genes still remains to be elucidated.

The selective inhibition of muscle gene expression with doxorubicin was limited to cardiac muscle cells. Doses 100 times the concentration used in cardiac cultures caused no alteration of either muscle or nonmuscle gene mRNAs in rat and human skeletal muscle cells. In animals, skeletal muscle has been shown to accumulate doxorubicin (28) to levels approximately half of that found in cardiac tissue, without associated toxicity (24). This has lead Olsen *et al.* (24) to propose that a metabolite of doxorubicin present in cardiac tissue may be responsible for the cardiac toxicity. Whether such tissue-specific differences in the metabolism of doxorubicin are responsible for differences in gene expression observed between our cardiac muscle cells and skeletal myoblasts is conjectural. However, our *in vitro* results closely parallel the clinical observation in humans that cardiac muscle is injured by doxorubicin at doses in which there are no effects on skeletal muscle.

Recognizing the possibility that these *in vitro* alterations in muscle gene expression with doxorubicin, although striking, might not correlate with *in vivo* effects of doxorubicin, we next treated adult rats with intravenous doxorubicin. As seen in Figs. 5 and 6, changes in muscle gene expression were also observed *in vivo*. Further, these changes precede any recognizable ultrastructural changes in cardiac tissue from doxorubicin-treated animals (Fig. 7). The resistance of skeletal muscle to doxorubicin was also shown *in vivo*: mRNAs from muscle genes were evaluated in skeletal and cardiac tissues from the same rats treated with doxorubicin. In all animals tested, the selective decrease in muscle genes was observed only in cardiac muscle (Fig. 6).

This report demonstrates that the cardiomyopathy produced by doxorubicin is associated with alterations in the expression of genes important for the structural integrity and enzymatic functions of cardiac muscle. Although no experimental system can be expected to model every aspect of the complex interaction between patient, disease, and drug, the fidelity of these *in vitro* results to *in vivo* changes suggests that selective inhibition of muscle gene expression may be an early event leading to the largely irreversible and sometimes fatal cardiomyopathy in patients treated with doxorubicin.

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