Time-Dependent and Tissue-Specific Accumulation of mtDNA and Respiratory Chain Defects in Chronic Doxorubicin Cardiomyopathy

Dirk Lebrecht, MS; Bernhard Setzer, PhD; Uwe-Peter Ketelsen, MD; Jörg Haberstroh, DVM; Ulrich A. Walker, MD

Background—Doxorubicin causes a chronic cardiomyopathy of unknown pathogenesis. We investigated whether acquired defects in mitochondrial DNA (mtDNA) and interconnected respiratory chain dysfunction may represent a molecular mechanism for its late onset.

Methods and Results—Rats were treated weekly with intravenous doxorubicin (1 mg/kg) for 7 weeks, starting at 11 weeks of age (group B). Controls received saline. Group C received doxorubicin identically to group B, but the course was started at 41 weeks of age. All rats were killed at week 48. Doxorubicin was also injected once, either 6 days (group D) or 2 hours (group E) before euthanasia. Heart and skeletal muscle were examined. Only group B rats developed a significant clinical, macroscopic, histological, and ultrastructural cardiomyopathy. Group B hearts had the lowest cytochrome c oxidase (COX) activity (24% of controls; \( P < 0.003 \)), the highest citrate synthase activity (135% of controls; \( P < 0.005 \)), and the highest production of superoxide. In group B, the respiratory subunit COXI, which is encoded by mtDNA, was reduced (\( P < 0.001 \)), as was mtDNA (49% of controls, \( P < 0.001 \)). Group C hearts differed from group B in their lower cardiomyopathy score (\( P < 0.006 \)), higher COX activity (\( P = 0.02 \)), and higher mtDNA content (\( P = 0.04 \)). Group B and to a lesser extent group C hearts contained deleted mtDNA. There was no detectable mitochondrial toxicity in group D and E hearts or in skeletal muscle.

Conclusions—In doxorubicin cardiomyopathy, mtDNA alterations, superoxide, and respiratory chain dysfunction accumulate long-term in the absence of the drug and are associated with a late onset. (Circulation. 2003;108:2423-2429.)

Key Words: cardiomyopathy ■ drugs ■ genetics ■ metabolism ■ pathology

The antitumor anthracycline antibiotic doxorubicin (adriamycin) is the drug of choice in the treatment of many malignancies, but its prolonged use is limited by an irreversible, dose-dependent, and progressive cardiomyopathy, which may become evident years after completion of therapy.1 The mechanism for the delayed onset and the molecular basis of the “dose memory” is not known.

We formulate the hypothesis that mitochondrial injury is acquired during acute doxorubicin exposure, accumulates with time in the absence of the anthracycline, and ultimately inhibits the bioenergetic capacity of the organelles (Figure 1). Doxorubicin enters mitochondria, inhibits the respiratory chain by binding to cardiolipin, or may interact with mitochondrial DNA (mtDNA), either directly or indirectly by generating reactive oxygen species (ROS).1 Our hypothesis hinges on the fact that respiratory chain dysfunction is associated with the mtDNA damage and can subsequently generate free radicals, which then in turn may either attack the respiratory chain itself or consecutively damage mtDNA.2 ROS may therefore close vicious circles composed of interconnected mtDNA and respiratory chain insults. Such vicious circles may continue to operate after acute doxorubicin treatment, account for the delayed manifestation of the cardiomyopathy, and represent or contribute to the molecular mechanism of dose memory.

Our study is the first to follow rats exposed to repeated doses of doxorubicin for several weeks. We demonstrate late-onset ultrastructural, genetic, and functional damage to the mitochondria in the absence of doxorubicin.

These new insights into the pathogenesis of doxorubicin cardiomyopathy may also contribute to the understanding of the cardiac damage secondary to ischemia and also in diseases caused by inherited defects of mtDNA.

Methods

Animals
Male Wistar rats (Charles River, Sulzfeld, Germany) were housed at a normal night-day rhythm, were fed a normal rat chow (SSnif...
R/M-H, Spezialdiäten), and received an intravenous port (Rat-O-Port, Uno Roestvaststaal) under anesthesia with isoflurane (Forene) at 10 weeks of age. The rats were divided into 5 groups (Figure 2).

Group A (n=7) served as control and received 7 intravenous injections of saline (300 to 700 μL) through the port beginning at 11 weeks of age. Groups B, C, D, and E received equivalent volumes of doxorubicin (1 mg/kg), which was freshly reconstituted in water from lyophilized powder (Pharmacia). Animals in group B (the cumulative long-term group, n=10) were given 7 weekly injections beginning at 11 weeks of age. Group C (the cumulative short-term group, n=9) received 7 weekly injections beginning at 41 weeks of age. Group D (n=8) and group E (n=6) animals received 1 single injection 6 days or 2 hours before euthanasia, respectively. Observations for mortality and clinical signs were carried out daily; body weights were recorded weekly. All animals were killed at 48 weeks of age by cervical dislocation immediately before organ collection and postmortem examination. The left gastrocnemius muscle, the left ventricle, and the apex were snap-frozen and cryopreserved in liquid nitrogen until subsequent analysis. Aliquots were fixed in glutaraldehyde (3%) for subsequent electron microscopy. All procedures were approved by the local review board for animal subjects.

**Histopathology**

Apical heart sections (4 μm) were stained with hematoxylin and eosin, and the severity and extent of lesions were scored on a qualitative/quantitative morphological grading scale. Left ventricle and skeletal muscle of 2 randomly selected rats from groups A, B, and C were examined by electron microscopy as described previously. The evaluating person was blinded to the treatment status of the rats.

**Enzyme Activity**

The activities of cytochrome c oxidase (COX), succinate dehydrogenase (SDH), and citrate synthase (CS) in freshly prepared tissue extracts were measured spectrophotometrically as described previously. COX and SDH are enzymes of the mitochondrial respiratory chain. COX is a multisubunit complex that is encoded partly by nuclear DNA (nDNA) and partly by mtDNA, whereas SDH is encoded entirely by nDNA. CS is an nDNA-encoded component of the Krebs cycle and is located in the mitochondrial matrix.

**mtDNA-Encoded Respiratory Chain Protein**

Subunit I of COX (COXI) is encoded by mtDNA, whereas subunit IV of COX (COXIV) is encoded by nDNA. COXI was quantified by immunoblot and normalized to the signal of a simultaneously used antibody against COXIV. The blots were also probed with an antibody (Research Diagnostics Inc) against GAPDH. The intensities of the signals were quantified densitometrically by use of Scion-image (Scion Corp).

**Wild-Type mtDNA**

Genomic DNA was extracted with phenol/chloroform from left anterior ventricle and gastrocnemius muscle, digested with the restriction enzyme XhoI, and quantified by Southern blot. mtDNA was probed with a 13.1-kbp, random-prime digoxigenin-labeled polymerase chain reaction (PCR) fragment spanning nucleotide positions 3192 and 16290 of rat mtDNA; the nDNA probe was directed against the multicopy 18S ribosomal DNA gene. DNA extracted from L6 cells and from control rat hearts was run on every gel as internal standard. The intensities of the wild-type mtDNA and nDNA signals were quantified by use of Scion-image, and wild-type mtDNA was normalized for nDNA content. The Southern blot procedure was also used to screen for large-scale mtDNA deletions.

**Detection of the Common mtDNA Deletion**

The sequence of normal rat mtDNA contains direct repeats between which base pairs may be deleted by slipped mispairing during replication. A 4834-bp deletion is the most frequent deletion and has been identified in senescent rats, similar to the age-related "common" 4977-bp deletion in humans. We probed for the common deletion in rats by amplifying 100 ng of genomic DNA with extradeletional primers in a PCR. By use of a short extension cycle (30 seconds), the deleted molecule was preferentially amplified as a 48-week-old male.

Figure 1. Proposed mechanisms of doxorubicin cardiotoxicity. Effects in presence of doxorubicin are depicted in gray; delayed effects of doxorubicin after it is washed out in black. Thickness of arrows represents relative contribution of each factor. In this model, initial insult that is below detection limit of our assays is perpetuated by vicious circles composed of ROS, mtDNA alterations, and interconnected respiratory chain insults.

Figure 2. Doxorubicin treatment of rats with weekly intravenous applications of doxorubicin (1 mg/kg; ▲) or of saline (▼). All animals were euthanized at 48 weeks of age (●).
12% lower than that of controls (P=0.004). From week 46 until death, group B rats lost ≈3% of body weight, whereas there was a 3% gain in the controls (P=0.004).

Unlike all other rats available for analysis, 6 of the group B animals had an increased respiratory rate after week 46, 6 had macroscopic evidence of myocardial dilatation, and 5 had pleural effusions. The livers of all animals were enlarged and dark red, with an engorged appearance. The macroscopic aspect of group C, D, and E organs did not differ from controls.

The myocardial ultrastructure of group C (Figure 3) was characterized by mitochondrial swelling and small vacuoles. In group B, interspersed vacuoles were also noted, but unlike group C, the myofibrillar lattice was in complete disarray, containing large clusters of intermyofibrillar mitochondria. The crystal architecture of the mitochondria was lost because of deposits of electron-dense material, which were never seen in group C. Skeletal muscle electron morphology did not differ among all groups and was without pathological lesions (not shown).

### Enzyme Activities
In the heart, the mean COX activity in group B was reduced to 24% (P=0.004, Table 2) of control values and was only 40% of group C (P=0.02). There was some reduction in the mean COX activity in group C compared with controls, but this reduction did not reach statistical significance (P=0.08). The SDH activity did not differ statistically from controls in group B (P=0.41) and

### Results

**Macroscopic and Microscopic Pathology**
Three of the 10 rats from group B died in weeks 38, 44, and 46, respectively. Postmortem examination revealed pleural effusions and a dilated myocardium in the first 2 animals. One of the 9 rats in group C died at week 48. All these animals were excluded from the analysis because we were unable to control for postmortem time.

Group B rats, unlike all other doxorubicin-treated groups, continuously gained body weight. The maximum medium body weight of group B was reached by week 46 and was ≈12% lower than that of controls (P=0.04). From week 46 until death, group B rats lost ≈3% of body weight, whereas there was a 3% gain in the controls (P=0.004).

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### Statistics
Group means were compared by unpaired t test or Wilcoxon analysis, as appropriate. Regressions were computed by nonlinear exponential regression analysis. All calculations were performed with the Sigma Plot 2000, version 6.0 (SPSS Inc) statistical package.

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### Table 1. Effects of Doxorubicin on Tissues

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<td>COX/COXIV*</td>
<td>100±17</td>
<td>68±15</td>
<td>81±15</td>
<td>96±15</td>
<td>98±13</td>
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<td>COXIV/GAPDH*</td>
<td>100±10</td>
<td>104±13</td>
<td>94±18</td>
<td>92±12</td>
<td>97±12</td>
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<tr>
<td>mtDNA/nDNA*</td>
<td>100±18</td>
<td>49±21</td>
<td>85±36</td>
<td>100±37</td>
<td>103±20</td>
<td>&lt;0.001</td>
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<td>*&quot;Common&quot; mtDNA deletion</td>
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<td>++</td>
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<td>–</td>
<td>N/A</td>
<td>N/A</td>
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<td>Superoxide*</td>
<td>100±29</td>
<td>838±270</td>
<td>499±150</td>
<td>118±42</td>
<td>107±25</td>
<td>&lt;0.001</td>
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NA indicates not applicable. The “common” mtDNA deletion was detected at low levels (+) in group C, at higher levels (++) in group B, but not (−) in groups A, D, and E. Values are group mean±SD.

*Values given as % of control mean.

459-bp product. The PCR product was gel-purified (Ultra Clean, MO BIO Laboratory) and sequenced (MWG Biotech).

**Superoxide Production**
Transverse sections were prepared for in situ imaging of superoxide with the oxidative fluorescent dye dihydroethidium (Sigma) as described elsewhere.9 The intensity of the fluorescence was quantified by use of Scion-image.

**Results**

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Unlike all other rats available for analysis, 6 of the group B animals had an increased respiratory rate after week 46, 6 had macroscopic evidence of myocardial dilatation, and 5 had pleural effusions. The livers of all animals were enlarged and dark red, with an engorged appearance. The macroscopic aspect of group C, D, and E organs did not differ from controls.

Compared with controls, the cardiomyopathy score (Table 1) was higher in group B (P<0.001) but not in group C animals (P=0.06). The cardiomyopathy score of group B was also higher than that of group C (P=0.006), whereas the scores of groups D and E did not differ statistically from the controls.

The myocardial ultrastructure of group C (Figure 3) was characterized by mitochondrial swelling and small vacuoles. In group B, interspersed vacuoles were also noted, but unlike group C, the myofibrillar lattice was in complete disarray, containing large clusters of intermyofibrillar mitochondria. The crystal architecture of the mitochondria was lost because of deposits of electron-dense material, which were never seen in group C. Skeletal muscle electron morphology did not differ among all groups and was without pathological lesions (not shown).
group C (P=0.7). The ratio of COX and SDH activities was reduced in group B (COX/SDH ratio, 23±24% of the control mean; P<0.001). In group C, the COX/SDH ratio was also lower than in controls (66±29%; P=0.02) but was higher than in group B (P=0.009). The COX/SDH ratio of groups D and E did not differ statistically from controls. The COX activity and the COX/SDH ratio correlated negatively with the cardiomyopathy score (P=0.002, r=−0.61, and P<0.001, r=−0.72, respectively; Figure 4, A and B).

Mean CS activity was increased by 35% in group B (P=0.005) and by 15% in group C hearts (P=0.03) compared with controls.

### mtDNA-Encoded Respiratory Chain Subunits

The mean COX/COXIV ratio was reduced in group B and group C hearts (68±15% and 81±15% of the control mean; P=0.003 and P=0.04, respectively) but did not differ statistically between groups C and B (P=0.15). The COXIV/GAPDH ratio did not differ statistically between groups (Table 1). The COX/COXIV ratio was inversely correlated (P=0.004, r=−0.47) with the cardiomyopathy score (Figure 4C) and positively correlated with both the absolute COX activity (P=0.04, r=0.37) and the COX/SDH ratio (P=0.04, r=0.36). These results are consistent with a link between reduced COX activity and a defect in the mtDNA-encoded COX subunits.

### mtDNA Content

The mean wild-type mtDNA content (mtDNA/nDNA ratio) in group B hearts was reduced compared with controls (49±21% versus 100±18%; P<0.001). The mtDNA/nDNA ratio in group C hearts was 85±21% of controls (P=0.33), which was higher (P=0.04) than that of group B. There was also no statistical reduction in the mean mtDNA/nDNA ratio of groups D and E (Table 1). Among all animals, the mtDNA/nDNA ratio was inversely correlated (P<0.001; r=−0.6) with the cardiomyopathy score (Figure 4D). The COX activity correlated positively with the mtDNA/nDNA ratio (P=0.004, r=0.48) and the COX/SDH ratio (P=0.005, r=0.48). These results suggest that mtDNA depletion contributes to the depression of COXI and the reduced COX activity.

### mtDNA Deletions

A 459-bp PCR product was amplified from all hearts of group B and group C and confirmed by sequencing to represent the common mtDNA deletion (Figure 5). The intensity of the PCR products from each group C heart was always lower than the lowest intensity of the PCR product from each group B heart. We failed to detect the common deletion in any of the control, group D, or group E hearts. No extra bands corresponding to deleted mtDNA molecules were identified in any sample of any tissue by Southern blot.

### Superoxide

The highest superoxide levels were identified in group B hearts (838±270% of control values; P<0.001). Superoxide was also elevated in group C (499±150%; P<0.001) but essentially normal in groups D and E (Table 1). Superoxide was higher in group B than group C (P<0.009). The superoxide levels correlated positively with the cardiomyopathy score (P<0.001, r=0.83; Figure 4E) and negatively with the absolute COX activity (P=0.004, r=−0.62), the COX/SDH ratio (P<0.001, r=−0.79; Figure 4F), the COXI/COXIV ratio (P<0.001, r=−0.57), and the mtDNA/nDNA ratio (P=0.002; r=−0.56).

### Skeletal Muscle

There were no macroscopic or microscopic abnormalities in skeletal muscle. In contrast to heart, there was no statistical difference in the mean COX/SDH ratio between group B and controls in skeletal muscle, and an increase of CS activity was not observed in group B. There was also no statistical difference in the COX/COXIV, the COXIV/GAPDH, and the mtDNA/nDNA ratios or in the superoxide levels between group B and controls in skeletal muscle. PCR did not identify any deletion in any animals of the control group, group B, or group C (Tables 1 and 2). Interestingly, the COX and SDH activity in group B was approximately twice that of control values (P=0.003 and 0.01, respectively).

### Discussion

We treated rats with doxorubicin and analyzed heart and skeletal muscle. The animals differed in the cumulative amounts of doxorubicin and in the time from the last doxorubicin administration until organ collection. Macroscopic, microscopic, and clinical evidence of myocardial damage was observed only in group B. Notably, the onset of the clinical symptoms did not occur immediately after the doxorubicin course but rather was delayed by several weeks. Minor and not quantitatively significant microscopic defects of the myocardium were also observed in rats receiving identical doses of doxorubicin but lacking a treatment-free interval from the last application of doxorubicin until organ...
collection (group C). Compared with other reports, the cumulative doses of doxorubicin in our study were lower.

Group B had also the most pronounced alterations in the function and composition of the respiratory chain, notably decreased COX activity and COXI expression, whereas nDNA-encoded mitochondrial components (SDH) were not functionally compromised or even enhanced (CS). This pattern of enzymatic alterations can also be observed in so-called “ragged-red fibers,” abnormal skeletal muscle fibers of patients with a cardiomyopathy caused by mtDNA mutations. Taken together, our results suggest a marked dysfunction of the mitochondrial genome.

Wild-type mtDNA levels were reduced exclusively in the hearts of group B, in which we also detected the highest levels of the common mtDNA deletion. This mtDNA mutation was observed previously with sensitive methods imme-
diately after repeated high doses of anthracyclines. The common mtDNA deletion is thought to arise from slip replication between two 16-bp direct repeats and has also been associated with oxidative stress and senescence. Aging, however, does not explain our observations, because all animals were age-matched. Instead, the common mtDNA deletion may be promoted by damaged mtDNA, which impedes replication by polymerase-β. Because of the absence of additional bands on the Southern blots, we must assume that the 4834-bp mtDNA deletion is present only at relatively low levels of heteroplasmy. The presence of such low levels of the common mtDNA deletion per se is unlikely to affect respiratory chain function to a significant degree. However, there are >200 direct repeats of ≥10 bp in rat mtDNA, and these repeats are candidate sites of additional mtDNA deletions arising from slip replication. The common mtDNA deletion may also be stochastically and heterogeneously distributed among individual cardiomyocytes, accounting for very high mutation levels in some fibers or in some fiber segments. Last, the mtDNA-encoded respiratory chain dysfunction may result from a combination of the quantitative and qualitative mtDNA defects.

Myocardial injury was most pronounced in group B, indicating that time is an important factor. Although it is formally possible that the hearts of young rats are more susceptible to doxorubicin, the group B rats developed clinical signs of cardiomyopathy not immediately but only weeks after drug discontinuation. Studies in humans indicate that advanced age is a risk factor for the long-term cardiotoxicity. Some reports suggest that doxorubicin at high doses also results in immediate mtDNA and respiratory chain damage and that some mtDNA mutations may persist for a long time because of inefficient repair, but our data indicate that the brunt of mtDNA damage does not arise immediately and is independent of continued drug exposure. What might be the underlying mechanism?

The direct interaction of doxorubicin with mtDNA is confined to the presence of anthracycline. Although doxorubicin accumulates in the heart, its pharmacokinetic behavior cannot explain the delayed onset of mtDNA alterations, because the intracardial half-life of the drug and its metabolites is only a few hours and exceeds the time interval from drug application to tissue sampling in groups B, C, and D by several fold. Free radicals are probably an important factor in the generation and maintenance of mutated mtDNA in doxorubicin cardiomyopathy, because mtDNA alterations can be prevented by exogenous and endogenous radical scavengers. Conversely, isolated defects in mitochondrial ROS scavenging systems are sufficient to cause a late-onset cardiomyopathy. Doxorubicin generates ROS directly by redox cycling, but an indirect source of oxygen radicals may be the respiratory chain, because doxorubicin binds with high affinity to cardiolipin, a phospholipid in the inner mitochondrial membrane that is required for full respiratory activity. Any inhibition of the respiratory chain increases ROS production. ROS are able to damage the lipid architecture of membranes, attack respiratory chain proteins, or affect mtDNA. Any of these injuries may compromise respiratory chain function and further increase ROS formation. Such vicious circles consisting of interconnected mtDNA and respiratory chain damage have also been suggested to represent a mechanism of aging and to explain the late onset of the cardiomyopathies associated with inherited mtDNA mutations. In both instances, deleted mtDNA molecules appear to increase in number with the passage of time. Similar mechanisms may be initiated during acute doxorubicin treatment and may subsequently carry on to operate even in the absence of continued drug exposure. The cardiomyopathy may become clinically and histologically apparent when the degree of combined respiratory chain and mtDNA insults exceeds a threshold. Group B rats (with the exception of 1 “outlier”) had <30% of residual COX/SDH activity, and a similar threshold level of COX deficiency has also been observed in cardiomyopathies associated with inherited mtDNA mutations. Notably, the outlier animal was also much closer to control values than its group peers with respect to the other parameters; it did not develop myocardial dilatation, and the respiratory rate appeared to be normal.

In our analysis, we focused on mtDNA and its respiratory chain gene products, but other mechanisms may contribute to the delayed onset of chronic doxorubicin cardiomyopathy. For example, proapoptotic factors of mitochondrial origin after increased superoxide production have recently been implicated in the execution of cardiomyocyte death. We also cannot rule out mutations in nuclear genes necessary for myocardial function, mtDNA maintenance, or mtDNA repair.

We failed to identify mtDNA damage or mitochondrial dysfunction in skeletal muscle and found a seemingly paradoxical upregulation of COX and SDH activity in this tissue. These findings may result from an adaptation to the reduced cardiac output in an attempt to maximize the arteriovenous oxygen extraction. Differences in the pathogenesis, degree, or time course of heart failure may contribute to the divergence between our observations and those of others.

Why is cardiac tissue the predominant target of doxorubicin toxicity? It has been suggested that the postmitotic nature of cardiomyocytes allows mtDNA mutations to accumulate in the inherited mitochondrial cytopathies, thus explaining the particular vulnerability of the heart. This argument, however, cannot be used, because skeletal muscle is also a postmitotic tissue. Doxorubicin accumulates in heart in high concentrations compared with other organs, suggesting that drug uptake might be an important factor. Doxorubicin also
complexes to cardiolipin, which is present in high concentrations in heart mitochondria.16 An NADH dehydrogenase able to initiate doxorubicin redox cycling and specific to heart mitochondria may promote ROS formation.15 Last, heart may have particularly low defenses against oxidative insults.18,21

Our data suggest that mtDNA alterations and respiratory chain defects, initiated during acute doxorubicin exposure and persisting or accumulating long-term and remote from the drug’s presence, may represent an important factor in the delayed onset of the cardiomyopathy. The elucidation of an association between mtDNA damage and cardiotoxicity might allow a dissociation of the antitumor effect of doxorubicin from its cardiotoxicity so as to increase the therapeutic index of this important anticancer drug. Our findings provide a conclusive model of the pathogenesis of chronic doxorubicin cardiomyopathy and may help in the rational development of new anthracyclines and cardioprotective radical scavengers.

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References
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