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Similar changes in cardiac morphology and DNA synthesis induced by doxorubicin and 4'-epi-doxorubicin

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Summary. *Doxorubicin is an antineoplastic agent whose clinical administration is limited by dose-dependent irreversible cardiomyopathy. Doxorubicin inhibits the rate of DNA synthesis in cultured rat myocardial cells after 1 h incubation with 16 μM, as is demonstrated by a decreased incorporation of [methyl-^{3}H]thymidine. An analogue of doxorubicin, 4'-epi-doxorubicin, also inhibits the rate of DNA synthesis within 1 h after treatment with 16 μM, to the same extent as doxorubicin-treatment of myocardial cells. Furthermore, similarity between doxorubicin and 4'-epi-doxorubicin in their effect on the myocardial thymidylate pool was also demonstrated by a significantly decreased incorporation of total [methyl-^{3}H]thymidine. The effect of doxorubicin on the rate of DNA synthesis in cultured rat skeletal muscle cells treated for 1 h with 16 μM was quantitatively the same as in myocardial cells. Light microscopy of doxorubicin- and 4'-epi-doxorubicin-treated myocardial cells and doxorubicin-treated skeletal muscle cells showed distinct nucleolar fragmentation and revealed no differences between the two drugs in their effect on either myocardial or skeletal muscle cells. Electron microscopy of myocardial cells following doxorubicin treatment showed increased nuclear pleomorphism and invaginations, along with a striking and distinctive clumping of nuclear chromatin. Furthermore, an apparent high density of the mitochondria due to an increased matrix volume and a concomitant decrease in the intermembrane compartment were observed. The results of this study indicate that doxorubicin-induced inhibition of cardiac DNA synthesis in cultured myocardial cells is nonpredictive of cardiotoxicity. The mechanism is at least bimodal, and the apparent minor toxicity of 4'-epi-doxorubicin compared with that of doxorubicin in clinical trials cannot be distinguished by a difference in the inhibition of DNA synthesis in the rat heart.*

Introduction

The anthracycline antibiotic doxorubicin (DX) is used in the treatment of many human neoplastic diseases [27]. In spite of the therapeutic effect of DX its clinical administration has been limited by a dose-dependent development of irreversible cardiomyopathy with severe congestive heart failure [10, 28] in addition to the side effects common to most cancer chemotherapeutic agents. The cardiotoxicity associated with the use of DX has long been recognized, but the mechanism of this toxicity is poorly understood. It is not clear why cardiac cells, which replicate at a very slow rate if at all, are also damaged by the very active antineoplastic drug DX. However, it has been suggested that interaction with nucleic acids in DNA and RNA, with cell membrane components and lipid peroxidation, plays a major role in the development of DX-induced cardiotoxicity [6, 7, 14, 16].

In an attempt to improve the therapeutic ratio of DX the development of analogues is being actively pursued, and 4'-epidoxorubicin (4'-epi-DX) is one of these analogues. It differs from DX only in being an epimer at the 4 position of the amino sugar. Early clinical trials suggested that it was less cardiotoxic than DX and had at least the same spectrum of antineoplastic activity [3].

Morphological changes, such as extensive myocytolysis concomitant with interstitial edema and fibrosis, myofibrillar dropout, nuclear damage, and degeneration of mitochondria and sarcoplasmic reticulum, have been described in animal studies and in cardiac cell cultures [13, 17, 25, 26].

The purpose of the present study was to evaluate the morphological changes in our cardiac cell culture after treatment with either DX or 4'-epi-DX and compare the myocardial cell damage with changes in skeletal muscle cells from subcultures; and to compare the effect of DX on DNA synthesis by monitoring the rate of incorporation of tritium-labeled thymidine in cardiac cells with that of 4'-epi-DX. To assess the predictive value of changes in the DNA synthesis, the effect of DX on DNA synthesis in cardiac cells was compared with that in cultures of rat skeletal muscle cells.

Materials

Neonatal Wistar rats (0–2 days old) were used. Hank's calcium- and magnesium-free balanced salt solution, buffered with 10 m*M* Hepes (Gibco BioCult), was used for washing during cell separation procedures. Crude collagenase, 0.05% (Boehringer Mannheim) was added during enzyme digestion.

The culture medium was Medium 199 (Gibco BioCult) with Hank's salts, 25 m*M* Hepes, and heat-inactivated horse serum, 10%.

Hank's solution and Medium 199 were supplemented with penicillin (75 units/ml) and streptomycin (75 μg/ml); all solutions were sterile.

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[Methyl-^{3}H]thymidine (^{3}H-TdR) with a specific activity of 77.1 Ci/mmol (New England Nuclear) was diluted in Medium 199 to a final activity of 0.125 μCi/ml incubation medium. DX and 4'epi-DX (kindly supplied by Farmitalia Carlo Erba, Milan, Italy) were dissolved in sterile water and further diluted in Medium 199 to the required concentration.

Methods

Cell cultures. A dissections and preparation procedures were carried out in a laminar-flow hood at 37° C, and cultures of both cardiac cells and skeletal muscle cells were established.

Cardiac cell cultures. The myocardial cells were isolated by stepwise enzyme digestion, as previously described by Demant and Wassermann [6]. The cells were plated in multi-dishes (Nunclon, Nunc, Denmark) at 0.5×10^6 cells/ml with 1 ml in each chamber for DNA synthesis assay, with 2 ml in petri dishes (Nunclon 240045, Nunc, Denmark) for light microscopy, and 6 ml in culture flasks (Nunclon 152094, Nunc, Denmark) for electron microscopy. The cultures were kept in a water-saturated (90%) incubator at 37° C (Hotpack, model 351820, Philadelphia, Pa, USA).

Skeletal muscle cell culture. The skeletal muscle cells were isolated by the same procedure as was used for the myocardial cells with few exceptions. Thigh muscles were isolated from skinned hind legs from 0 to 2-day-old rats, and the enzyme digestion time was 30 min for each of five cycles. The pooled supernatants containing skeletal muscle cells were filtered through a double layer of sterile gaze before centrifugation and resuspension in Hank's solution. To separate the muscle cells from nonmuscle cells the cell suspension in culture medium was transferred to a petri dish for cell cultures for 20 min. The viable cells were counted in a hemotocymeter after trypan blue staining.

The cells were plated in multi-dishes and petri dishes at the same viable cell density as the cardiac cells.

DNA synthesis. After 68 h in culture the cells were incubated with either DX or 4'-epi-DX (16 μ*M*) for 1 h. After this incubation the DX and 4'epi-DX medium were discarded and the cells for DNA synthesis assay were reincubated with ^{3}H-TdR. Untreated controls from each cell culture were run in parallel in a similar way.

At specific times after the DX or 4'-epi-DX treatment both controls and treated cells were incubated in duplicate with 0.125 μCi ^{3}H-TdR/ml for 15, 30, 60, 90, 120, 150, 240, and 300 min. At the end of the incubation time the radioactive medium was aspirated and the cells washed rapidly three times with a 0.9% NaCl solution (4° C). At this point the extracellular space was found to be less than 0.3% by inuline. The acid-soluble material (AS) [1] was extracted twice into 1 ml cold 4% trichloracetic acid (TCA) for 10–15 min. Scintillation liquid, 2.5 ml (Opti-Fluor, Packard), was added to 500 μl of the pooled acid extracts.

The TCA-extracted cells were washed twice with ethanol and dissolved in 500 μl 0.5 *N* NaOH. The amount of ^{3}H-TdR in this solute that originated in DNA-incorporated ^{3}H-TdR [1] was measured after addition of 2.5 ml scintillation liquid (Opti-Fluor, Packard). The radioactivity was measured in an LKB 1216 Rackbeta liquid scintillation counter. The efficiency was 19% estimated with ^{3}H-toluene as internal standard.

Cellular protein was chosen as a reproducible parameter to which ^{3}H-TdR incorporation into DNA and acid-soluble material was standarized.

Protein was determined on the dissolved cells using the method of Lowry et al. [15].

Statistical analysis was performed by Student's *t*-test.

Light and electron microscopy. The cell cultures intended for light microscopy were treated with DX or 4'-epi-DX (16 μ*M*) for 1 h. After drug treatment the cells were washed three times with a 0.9% NaCl solution (4° C) and fixed in Lillie's fixative for 30 min, dehydrated with ethanol and embedded in Aquamount. Controls and DX-exposed and 4'-epi-DX-exposed cultures were examined with phase-contrast and bright field optics following PAS staining [19] to distinguish muscle cells from fibroblasts and endothelial cells and to determine the relative number of surviving muscle cells and follow the morphological changes at the light microscopic level after exposure to the drugs.

Cultures of myocardial cells for electron microscopy were treated with DX (16 μ*M*) for 4 h. After the treatment and after washing with 0.9% NaCl solution (4° C) the cells were fixed in 2% glutaraldehyde in a 0.1 *M* cacodylate buffer for 30 min. After fixation the cells were scraped off the plates and centrifuged in cadodylate buffer for 25 min at 1600 *g*. The pellets were treated with 2% OsO_4 in 0.1 *M* cacodylate buffer, pH 7.3 for 1 h at 4° C, rinsed briefly in buffer followed by distilled H_2O, and block-stained for 1 h at room temperature in 1% uranyl acetate in H_2O. The specimens were dehydrated in increasing concentrations of ethanol and embedded in Epon. Untreated controls were processed in parallel.

Areas suitable for electron microscopy were localized in 1 μm sections stained with toluidine blue. Thin sections 20–30 nm thick were stained with uranyl acetate and lead citrate and examined in a Jeol 100 CX electron microscope.

Results

Myocardial cells

DNA synthesis. In the untreated cultures of myocardial cells a linear correlation between ^{3}H-TdR incorporation and exposure time during the 5-h incubation period was demonstrated. The rate of DNA synthesis estimated by the rate of ^{3}H-TdR incorporation, was 0.85 ± 0.14 fmol ^{3}H-TdR/h (Fig. 1). The incorporation of ^{3}H-TdR in DNA in cells pretreated with either DX or 4'-epi-DX also revealed a linear correlation (Fig. 1). The rates of ^{3}H-TdR incorporation in the DX- and 4'-epi-DX-pretreated cells were significantly reduced compared with that obtained in the untreated controls (Table 1, $P_{DX} < 0.05$, $P_{4\text{-epi-DX}} < 0.01$). However, no significant difference was observed between the DNA synthesis in DX- and 4'-epi-DX-pretreated cells.

Cellular thymidine compartments. Non-DNA-bound ^{3}-H-TdR, the acid-soluble compartment, increased to a maximum after about 30 min exposure to ^{3}H-TdR in the untreated cells (Fig. 2). The amount of ^{3}H-TdR in the acid-soluble compartment at steady state (4 h) was 0.15 ± 0.03 fmol ^{3}H-TdR. The amount of ^{3}H-TdR in the acid-sol-

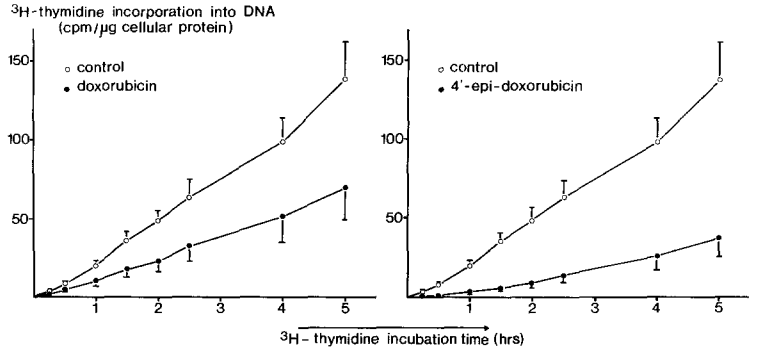


Fig. 1a Incorporation of ^{3}H-thymidine (specific activity 77.1 Ci/mmol) into DNA in control (o) and *(left)* doxorubicin-treated (●), *(right)* 4'-epi-doxorubicin-treated (●) myocardial cell cultures from neonatal rats. Cultures were incubated with doxorubicin or 4'-epi-doxorubicin (16 µ*M*) for 1 h before ^{3}H-thymidine incubation. Each *point* represents mean and SEM each *bar*, of ^{3}H-thymidine incorporation into DNA per microgram of cellular protein in 11 individual control experiments, 7 with doxorubicin, or 6 with 4'-epi-doxorubicin

Table 1. The rate of DNA synthesis estimated by the rate of ^{3}H-thymidine incorporation in DX- and 4'-epi-DX-treated myocardial cell cultures and in doxorubicin-treated skeletal muscle cell cultures

	Myocardial cell cultures fmol ^{3}H-TdR/h	P[c]		Skeletal muscle cell cultures fmol ^{3}H-TdR/h	P
Control	0.85 ± 0.14[a] (11)[b]			0.66 ± 0.05 (5)	
		P < 0.05			P < 0.001
DX (16 µ*M*, 1 h)	0.43 ± 0.13 (7)		P ± 0.01	0.19 ± 0.03 (5)	
		NS[d]			
4'-Epi-DX (16 µ*M*, 1 h)	0.25 ± 0.08 (6)			–	

a Mean and SEM
b Number of experiments
c Statistical significance based on Student's t-test
d NS, not significant

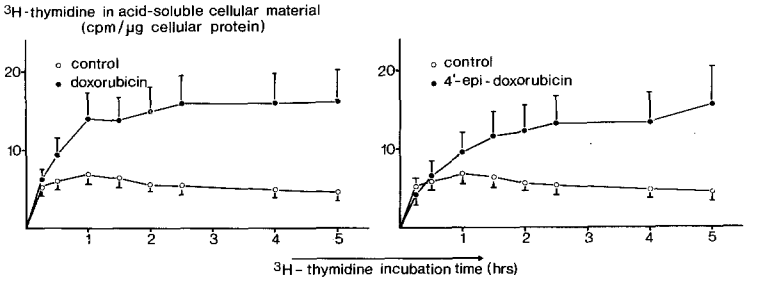


Fig. 2. Incorporation of ^{3}H-thymidine (specific activity 77.1 Ci/mmol) into acid-soluble cellular material in control (o) and *(left)* doxorubicin-treated (●), *(right)* 4'-epi-doxorubicintreated (●) myocardial cell cultures from neonatal rats. Cultures were incubated with doxorubicin or 4'-epi-doxorubicin (16 µ*M*) for 1 h before ^{3}H-thymidine incorporation. Each *point* represents mean and each *bar*, SEM of ^{3}H-thymidine in acid-soluble cellular material per 7 microgram of cellular protein in 11 individual control experiment, 7 with doxorubicin, and 6 with 4'-epi-doxorubicin

Table 2. Amount of ^{3}H-thymidine in various cellular compartments in doxorubicin- and 4'-epi-doxorubicin-treated myocardial cell cultures after 4 h incubation

	DNA fmol ^{3}H-TdR	Acid-soluble fmol ^{3}H-TdR	Calculated[c] total fmol ^{3}H-TdR	Measured total fmol ^{3}H-TdR
Control	2.99 ± 0.46[a] (11)[b]	0.15 ± 0.03 (11)	3.14	2.76 ± 0.63 (5)
DX (16 µ*M*, 1 h)	1.57 ± 0.48 (7)	0.48 ± 0.11 (7)	2.05	–
4'-Epi-DX (16 µ*M*, 1 h)	0.81 ± 0.27 (6)	0.41 ± 0.11 (6)	1.22	1.82 ± 0.51 (5)

[a] mean and SEM;
[b] number of experiments;
[c] amount of ^{3}H-TdR in DNA plus in acid-soluble compartment

uble compartment was significantly greater in both DX- and 4'-epi-DX-treated cells (Table 2: $P_{DX} < 0.05$, $P_{4'\text{-epi-DX}} < 0.05$) and the time needed for steady state to be reached was significantly longer than in the untreated cells (Fig. 2: $P < 0.001$). There was no significant difference between the level of steady state in DX- and that in 4'-epi-DX- pretreated cells.

The calculated total amount of ^{3}-H-TdR in the myocardial cells is derived from the DNA-bound and the acid-soluble ^{3}H-TdR and was significantly reduced, to the same level, in DX- and 4'-epi-DX-treated cells (Table 2: $P < 0.05$). The total amount of cellular ^{3}H-TdR was measured directly in 4'-epi-DX-treated cells and was the same as the calculated amount (Table 2).

Skeletal muscle cells

A linear correlation between DNA incorporation of ^{3}H-TdR in untreated skeletal muscle cells and exposure time was demonstrated (Fig. 3), and the rate of DNA synthesis was the same as that obtained in myocardial cells (Table 1). The rate of DNA synthesis was significantly reduced by DX, to the same extent as in myocardial cells (Table 1: $P < 0.001$).

The acid-soluble compartment of ^{3}H-TdR was also increased by the same extent as in the treated myocardial cells (Fig. 2: $P < 0.05$).

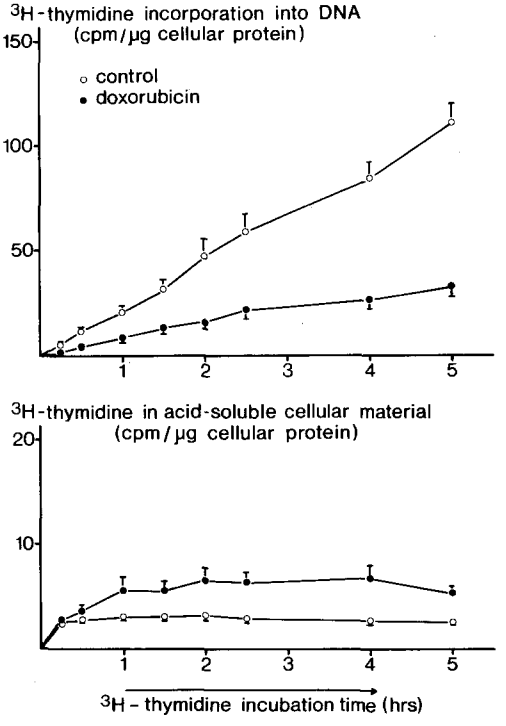


Fig. 3. Incorporation of ^{3}H-thymidine (specific activity 77.1 Ci/mmol) *(upper part)* into DNA and *(lower part)* into acid-soluble cellular material in control (o) and doxorubicin-treated (●) cultured skeletal muscle cells from neonatal rats. Cultures were incubated with doxorubicin (16 µ*M*) for 1 h before ^{3}H-thymidine incubation. Each *point* represents mean and each bar, SEM of ^{3}H-thymidine per microgram of cellular protein in five individual control experiments and five with doxorubicin

Light and electron microscopy

The purity of both cardiac and skeletal muscle cell cultures with regard to muscle cells evaluated after PAS staining was about 80%. Comparisons of the results obtained from control cultures, DX-exposed cultures, and 4'-epi-DX-exposed cultures in light microscopy showed that marked changes had occurred in the nuclear morphology of both cardiac and skeletal muscle cells after drug exposure (Fig. 4). The nuclei appeared to te pleomorphic and exhibited a distinct nucleolar fragmentation and segregation. Nuclei of nonmuscle cells (fibroblasts and a few endothelial cells) showed similar changes.

In the electron microscope cardiac muscle cells from control and DX-exposed cultures exhibited fine-structural features similar to those recently reported in a detailed quantitative study by Tobin and Abbot [25]. The ovoid nuclei of control cultured myocardial cells contained distinct nucleoli with normal pale-staining, granular and fibrillar components, and exhibited a narrow band of peripheral envelope (Fig. 5). The mitochondria, which occupied a major portion of the cell volume, showed folded mitochondrial cristae separated by a distinct intermembrane space.

Exposure to DX caused clear-cut changes in nuclear and mitochondrial morphology, whereas other intracellular components and organelles showed no obvious qualitative differences from the control situation. The nuclei in DX-exposed muscle cells showed pronounced differences in shape and chromatin pattern. The nuclei in treated cells were frequently elongated with a wrinkled nuclear envelope (Fig. 6). The peripheral chromatin was no longer in contact with the inner membrane of the nuclear envelope and exhibited a characteristic clumping (Figs. 6 and 7). Mitochondrial profiles appeared to be more frequent after DX exposure. Furthermore, the density of the individual mitochondria was increased owing to an expansion of the matrix on the expense of the intermembrane compartment (Fig. 6 and 7).

Discussion

The nuclear changes observed in our cultured myocardial cells, involving nucleolar fragmentation and segregation along with decreased incorporation of ^{3}H-TdR, indicating inhibition of DNA synthesis, have already been described [2, 7, 11, 12, 13, 21, 22, 24]. In contrast to the findings of Lampidis et al. [12, 13], we found no nuclear vacuolization in the DX-treated cells. Furthermore, we did not observe hypertrophy and swelling of the sarcoplasmic reticulum or an increase of the number and total extension of the gap junction [17]. In agreement with the findings of Tobin and Abbot [25], we found mitochondrial hypertrophy and an increase in the proportion of pleomorphic nuclei compared with controls. In addition, we observed an apparent high density of the mitochondria owing to an increased matrix volume and a concomitant decrease in the intermembrane compartment.

The mitochondrial structure and function are intimately correlated. The positively charged amino group of DX has a high affinity for the negatively charged cardiolipin [20], which constitutes about 20% of the lipids in the mitochondrial cristae, and this reaction is probably irreversible. Furthermore, DX-induced production of free radical

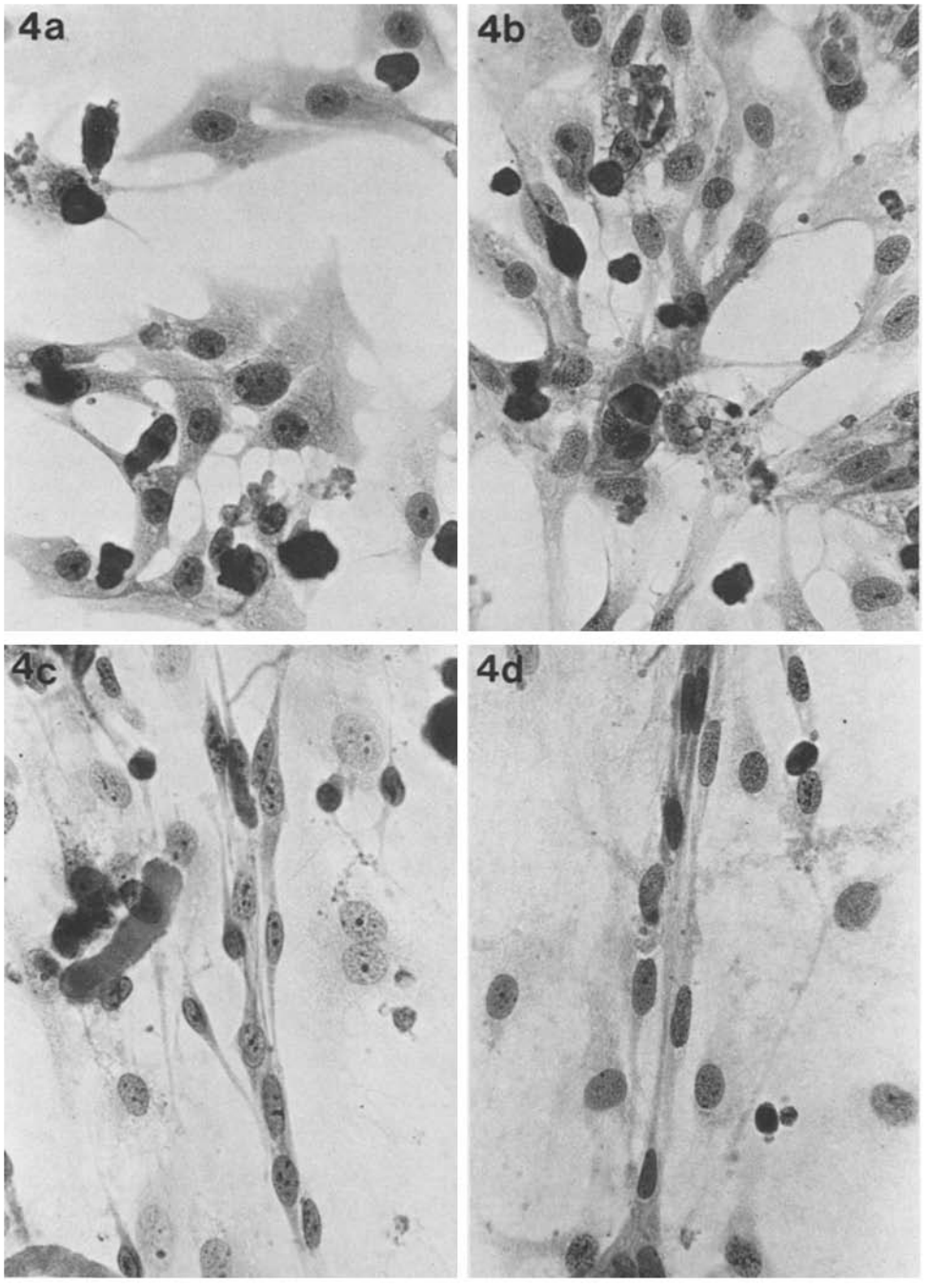


Fig. 4 a– d. Photomicrographs of cardiac **(a, b)** and skeletal muscle **(c, d)** cultures fixed 60 h after plating. The cells shown in **b** and **d** were exposed to DX during the last hour of the experiment. Note the characteristic difference in nuclear morphology between cells from control cultures **(a, c)** and DX-exposed cultures. PAS: ×495)

metabolites may lead to lipid peroxidation of the mitochondrial membrane [20]. The interference of DX with oxidative phosphorylation [9], the reduced respiratory activity [5], and the lower intracellular ATP level [23] demonstrate that DX causes a decrease in energy generated in the mitochondria. DX-induced impairment of the mitochondrial capacity to generate ATP may result in reduced phosphorylation of thymidine to the stepwise formation of thymidine triphosphate, which may be insufficient for incorporation into DNA. Thus, a simulated inhibition of ^{3}H-TdR incorporation into DNA by way of an effect on the thymidylate pools may be the consequence. Presumably, phosphorylation of thymidine is affected by DX along with inhibition of the DNA synthesis as a result of a direct physical intercalation with the paired stacked bases of double-stranded DNA.

The calculated total cellular amount of ^{3}H-TdR in the DX- and 4'-epi-DX-treated cells was significantly reduced, and steady state in the acid-soluble compartment was obtained significantly later in the untreated cells. These two phenomena may be a result of an affected thymidilate pool by an insufficient phosphorylation of thymidine and

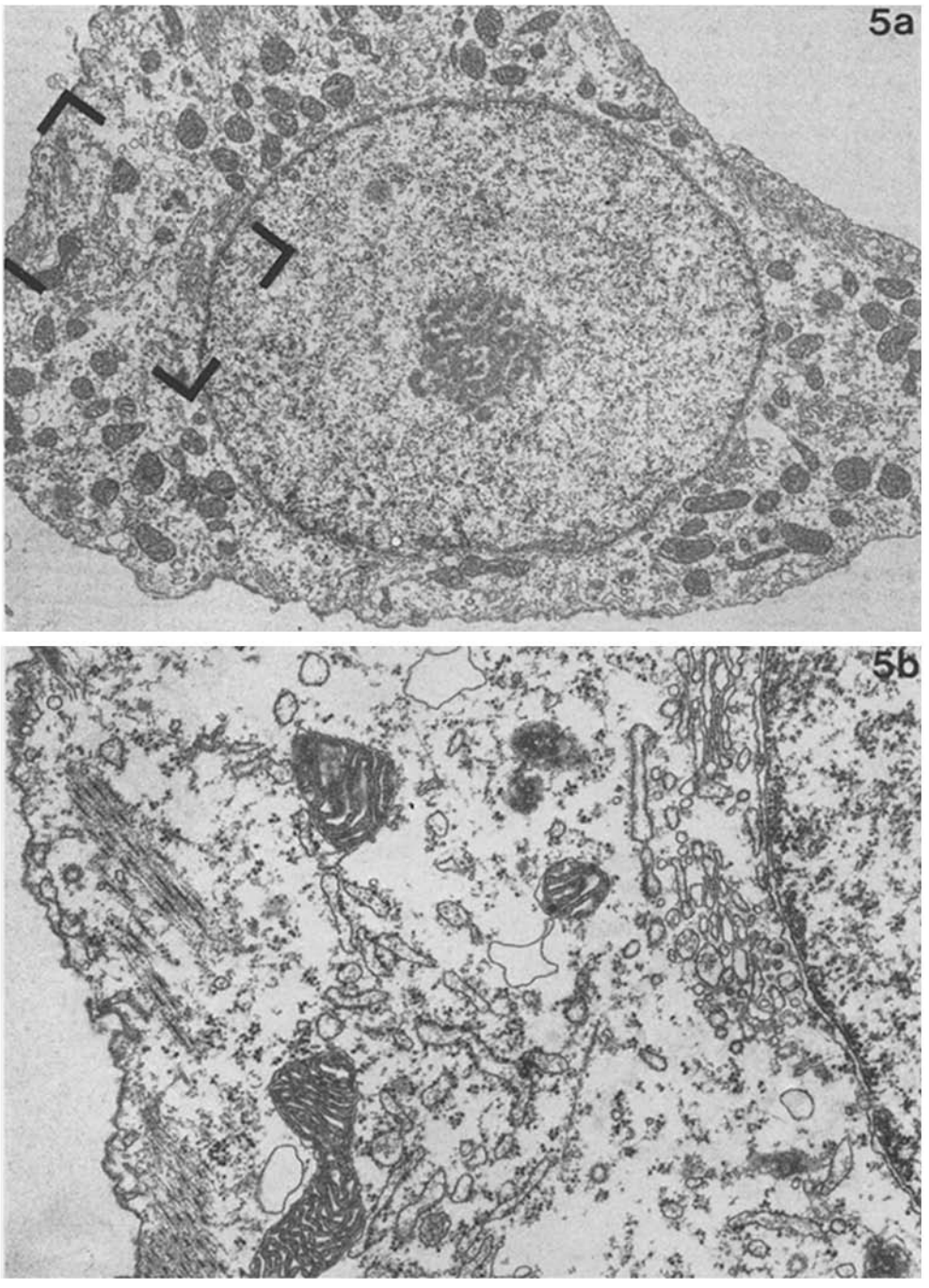
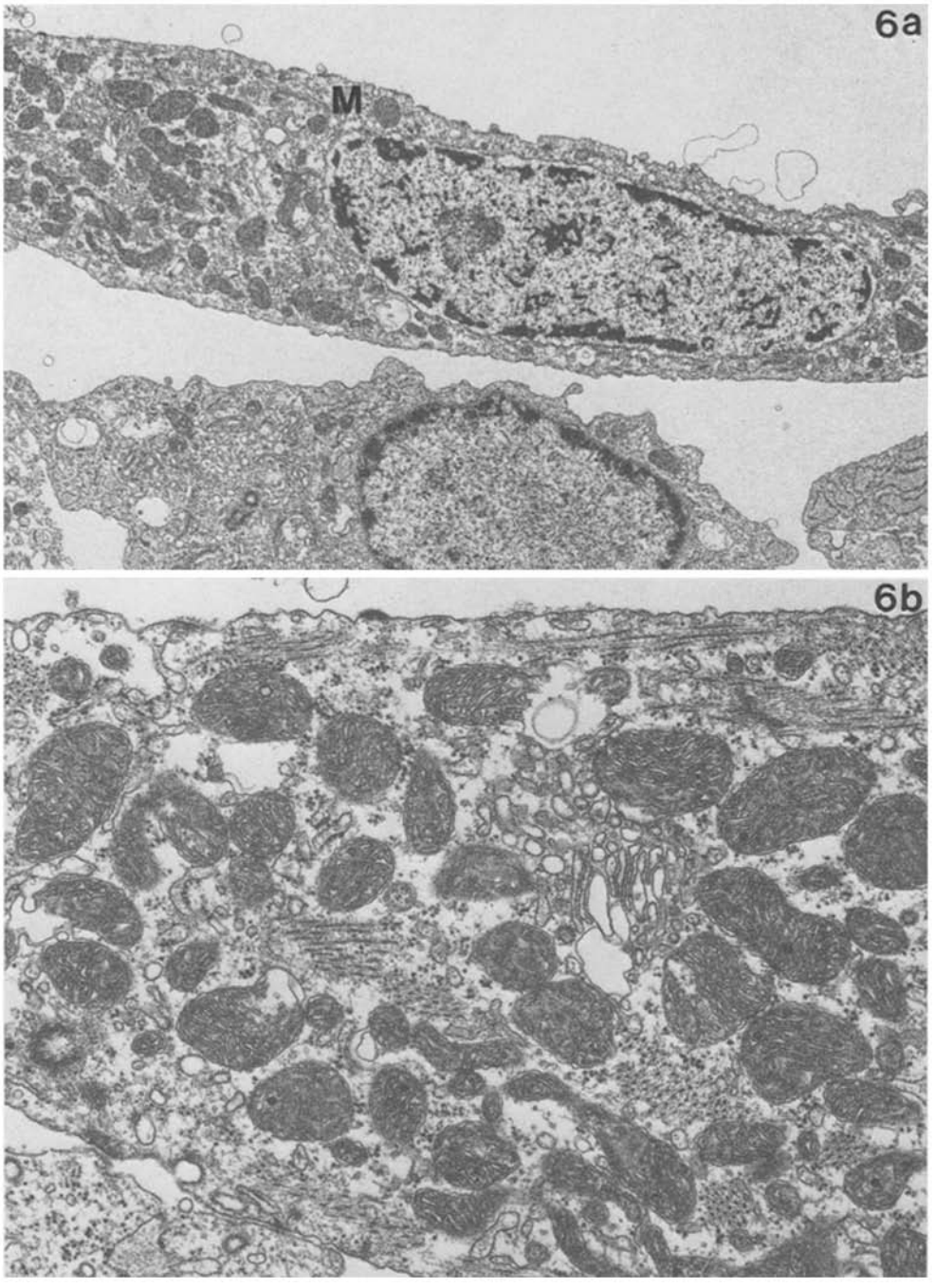


Fig. 5 a, b. Thin section micrograph of a cardiac myoblast at low (**a**; ×7300) and high (**b**; 26000) magnification after 72 h in culture. **a** The peripheral heterochromatin is associated with the inner membrane of the nuclear envelope and a distinct nucleolus is present. **b** A few bundes of myofilaments and some cisterns of sarcoplasmic reticulum and of the Golgi complex are depicted. Note the characteristic intermembrane space in the mitochondria.

possibly a decreased uptake of extracellular ^{3}H-TdR into the cells. Both mechanisms could be due to a primary effect of DX or combined with a negative feedback inhibition of thymidine kinase by thymidine triphosphate in consequence of a decreased incorporation into DNA. However, an unambiguous answer cannot be obtained on the basis of our data. The increased amount of non-DNA-bound ^{3}H-TdR, the acid-soluble compartment, observed in the DX- and 4'-epi-DX-treated myocardial cells is not consistent with recent results of Formelli et al. [8], who studied the effects of DX on DNA synthesis in mouse and rat heart in vivo. They measured the total acid-soluble radioactivity and the radioactivity in fractions of thymidine monophosphate, diphosphate, and triphosphate in hearts from DX-treated mice and found no appreciable differences between control and treated tissue. However, a direct extrapolation from in vitro to in vivo data is not advisable. There is, however, a fundamental difference in the



Figs. 6 a. Thin section at low magnification of a cardiac myoblast *(M)* after 4 h exposure to DX. Note the clumping of the peripheral chromatin in the elongated nucleus of the myoblast and the numerous dense mitochondria (× 7300). **b.** Some of the dense mitochondria shown at high magnification. Note the increased volume of matrix compared with mitochondria from control cultures. (× 26000)

^{3}H-TdR pulse times used by Formelli et al. [8] and those used in our experiments. Formelli et al. [8] chose a 10-min pulse time after an SC injection of ^{3}H-TdR, whereas no appreciable differences between the amount of ^{3}H-TdR in acid-soluble cellular materials were observed by us with the same pulse time either in controls or in treated cells (Fig. 2).

In histopathological studies of the myocardium in rabbits the cardiac toxicity of 4'-epi-DX is suggested to be less than that of DX, while the morphological changes were qualitatively the same [4].

In the present study, 4'-epi-DX showed both qualitatively and quantitatively the same effect as DX on the estimated rate of DNA synthesis and nuclear changes in the myocardial cells when the cells were exposed to equal doses, and these findings are in agreement with those of Plumbridge and Brown [18], who concluded that the apparently reduced cardiotoxic effect of 4'-epi-DX in vitro compared with that of DX is probably not due to a difference in the interaction with DNA. The difference in cardiotoxic effect may be caused by different pharmacokinetic properties. However, our observations and the results of

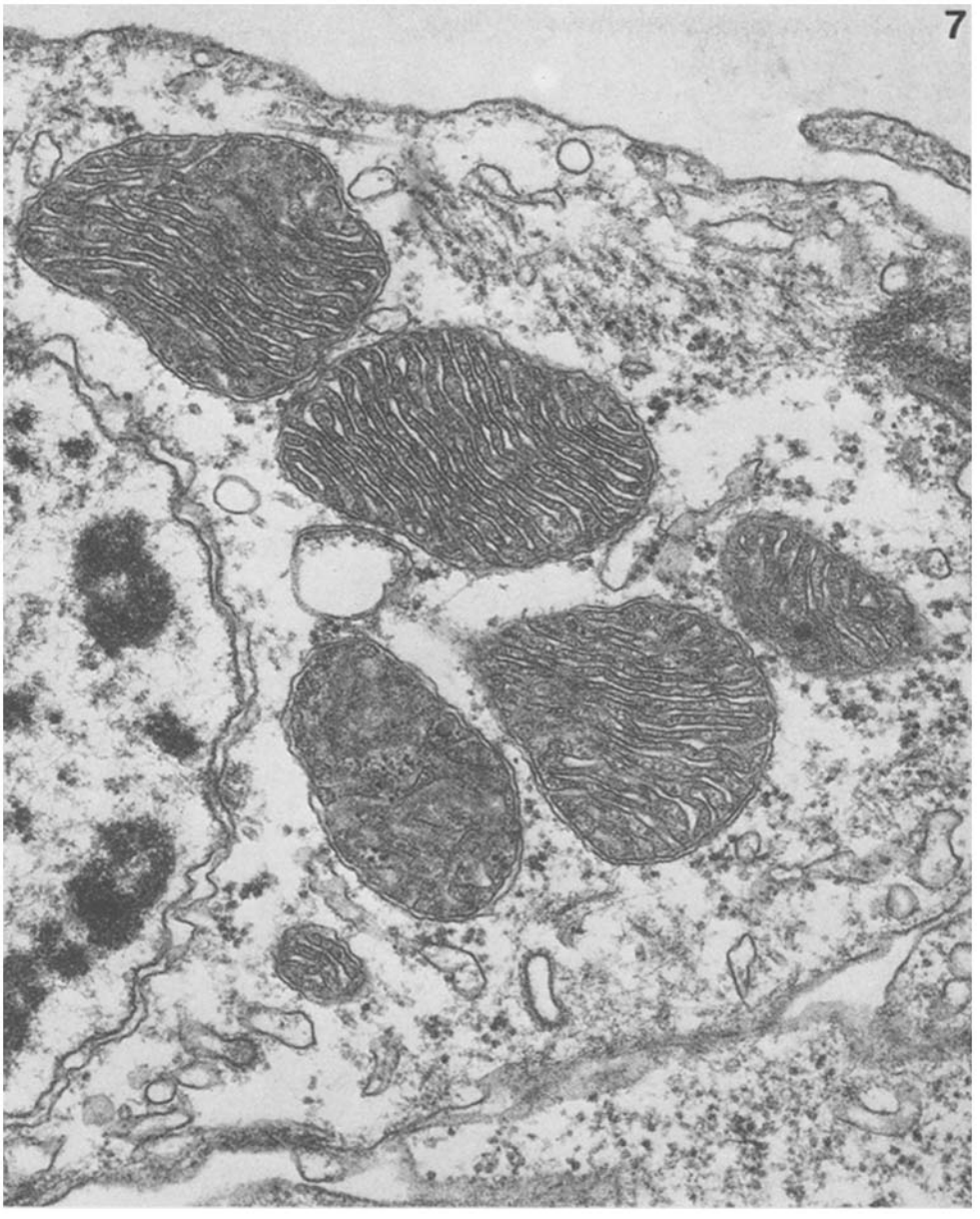


Fig. 7. High magnification of a DX-exposed cardiac myoblast, showing clumping of the peripheral chromatin which is no longer in contact with the inner membrane of the now folded nuclear envelope. The apparent high density of the mitochondria is at least partly due to an increased matrix volume and a concomitant decrease in the intermembrane compartment. × 49000

Plumbridge and Brown [18] do not exclude a lesser toxicity of 4'-epi-DX observed in preclinical and clinical trials.

Finally, we found that the rate of DNA synthesis in cultured skeletal muscle cells was significantly reduced by DX to the same extent as seen in myocardial cells (Table 1), indicating a lack of relationship between inhibition of DNA synthesis and cardiotoxicity of DX in cultured cell systems. Morphological changes evaluated by light microscopy were similar to those seen in DX-treated myocardial cells. There is therefore no correlation between the rate of DNA synthesis measured by ^{3}H-TdR incorporation in cultured myocardial cells and the anthracycline-induced cardiotoxicity. However, the early morphological changes and the inhibition of DNA synthesis following DX treatment may be of importance for the development of DX-induced cardiomyopathy, although it does not have any predictive value.

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