Cancer Chemother Pharmacol (1985) 15: 26–30

Cancer Chemotherapy and Pharmacology

Lysosomal alterations in heart and liver of mice treated with doxorubicin*

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Summary. This study was carried out to evaluate the influence of long-term treatment with doxorubicin (DXR) (4 mg/kg IV for 5 weeks) on heart and liver lysosomes of mice.

We evaluated the variations in both total and "sedimentable" enzyme activity of cathepsin D, which is the major endopeptidase of myocites and probably involved in physiologic and pathologic degradation of actomyosin and mitochondria, and that of acid phosphatase, which is more prominent in interstitial cells.

Our results show that marked changes occur in both total and sedimentable enzyme activity of cathepsin D in the heart of treated animals and to a lesser extent in the liver.

In contrast, no modification of either total or sedimentable acid phosphatase was seen in either organ.

The effects we observed are much more marked for cardiac cathepsin D; this is in good agreement with the cardiac specificity of DXR-induced cardiotoxicity with long-term administration and suggests that lysosomes could play a role in the pathogenesis of this phenomenon.

Introduction

Many hypotheses have been proposed about the mechanism of anthracycline-induced chronic cardiotoxicity.

Table 1 lists the main hypotheses that have been tested in connection with this phenomenon [2, 3, 6, 27]. None of these studies has clearly identified the mechanism of this action, but it has been possible to define the morphological characteristics of this cardiomyopathy by light and electron microscopy. Solcia et al. [23], Mettler et al. [15], and Jaenke [12] have observed by electron microscopy that cardiac lesions in mice, rats, and rabbits, respectively, are quite similar: Myofibrils are disrupted with lysis of the myofilaments, and mitochondria are reduced in overall size and affected by the formation of electron-dense concentric lamellae and altered matrix density, whereas tubules and cisterns of sarcoplasmic reticulum and the membrane system of the T tubules show less severe damage, mainly consisting in pronounced swelling.

* Supported by CNR (National Research Council) contract No. 83.00923.96

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Table 1. Main hypothesis on mechanism of DXR-induced cardiotoxicity

Impairment of nucleic acids synthesis [2, 25]
Free radicals generation [3]
Drug-induced histamine release [6]

The greater severity of the lesions of the mitochondria and myofibrils led us to hypothesize that lysosomes could play a role in the cardiotoxicity of anthracyclines since among other hydrolases they contain cathepsin D, an endopeptidase that exerts a pronounced effect on both myofibrils and mitochondria [1, 11, 21]. On the basis of these data we examined mice subjected to long-term treatment with doxorubicin (DXR) to evaluate the total enzyme activity and the intra- and extralysosomal distribution, i.e., the so-called sedimentability [26], of cathepsin D and acid phosphatase. We chose these two enzymes because the former is the major endopeptidase in myocites, whereas the latter is more prominent in interstitial cells [25], so that any difference in the behavior of these two enzymes in treated animals could be used to detect whether treatment affects one or both types of cells.

Materials and methods

Female mice (Charles River CD-1 strain COBS) weighing 25 ± 2 g were divided into two groups of ten animals each; the first group functioned as controls and received saline alone, while the second received DXR 4 mg/kg IV dissolved in saline, weekly for up to 5 weeks.

Animals were deprived of food overnight before sacrifice. They were killed by cervical dislocation at least 12 h after the first treatment.

Hearts and livers were rapidly removed, after which the atria of the heart were cut off and the pooled organs were rinsed, blotted on filter paper, weighed, and minced with cold scissors. The minces were suspended in 10 vols. homogenization medium (2°C) containing 0.25 M KCl and 1 mM EDTA buffered with 0.05 M Tris (pH 7.4) and homogenized in a Willelms Polytron PT-10 (Kinematica Kriens-Luzern) set to rotate at a slow speed so that lysosomes would remain intact as far as possible [13]. The homogenate was centrifuged at 350 g for 5 min and the pellet, which contained nuclei, tissue fragments, and cell debris, was discarded. An aliquot of the 350 g supernatant

was vigorously homogenized in an aqueous solution of 0.2% Triton X-100 and assayed for cathepsin D and acid phosphatase by the methods described by Barrett [4] and for proteins by the Lowry technique [14]. The assay for cathepsin D involved measurement of TCA-soluble Folin-reactive products of hemoglobin digestion at pH 3.2 and 45° C (Barrett's method II); confirmation that proteolitic activity was attributable only to cathepsin D was obtained by the demonstration of almost 100% inhibition in the presence of 0.1 μg/ml sodium pepstatin, which specifically inhibits cathepsin D in such tissue homogenates [5].

The assay for acid phosphatase involved the measurement of p-nitrophenol released from nitrophenilphosphate at pH 4.7 and 37° C after inhibition of nonlysosomal phosphatases with sodium acetate [4].

For measurement of sedimentable vs nonsedimentable enzyme activity, the remainder of the 350 g supernatant was respun at 40,000 g for 20 min (Spinco Model L50, Beckman). The second pellet, which contained intact lysosomes, mitochondria, and other organelles, was resuspended in 0.2% Triton X-100 in 0.25 M KCl and vigorously homogenized to release lysosomal enzyme activity. This fraction, termed 'sedimentable' (ML) was assayed for cathepsin D and acid phosphatase as described above. The 40000 g supernatant, termed 'nonsedimentable' (PLS) was assayed similarly.

Results are expressed according to the formula:

$$Sedimentability = \frac{ML \times 100}{ML + PLS},$$

where ML (mitochondrial-lysosomal fraction) is the enzyme activity detected in the 40,000 g pellet; and PLS (postlysosomal supernatant) is the enzyme activity detected in the 40,000 g supernatant.

Samples were maintained at a constant temperature of 4 °C until assay.

Statistical analysis. Differences in the enzyme activities, sedimentability, and proteins between control and treated animals were analyzed for statistical significance by Student's t-test for unpaired samples, the 0.05 level of probability being taken as significant [22].

Results

Table 2 shows the total activity of cathepsin D in the heart and liver (taken for comparison) of DXR-treated animals compared with controls. At the same doses as are used to evoke morphological lesions [10], the drug initially caused a reduction of cathepsin D in the heart, which became statistically significant on day 3 after the first treatment; afterwards this trend reversed and the total enzyme activity reached higher values in treated animals than in controls, the differences being statistically significant after 5 weeks of treatment.

The liver initially showed a similar reduction of cathepsin D on day 3 after the first treatment, but in contrast to the course in the heart, as treatment progressed any difference disappeared.

Table 3 shows the total activity of acid phosphatase: In contrast to cathepsin D it was never found to differ for either organ between treated animals and controls.

Table 4 shows the variations in cathepsin D sedimentability: again a characteristic behavior was seen in the heart: Cathepsin D sedimentability increased conspicuous-

Table 2. Effect of DXR (4 mg/kg IV weekly) on cathepsin D total activity in mouse heart and liver

Time of treatment	Heart		P	Liver		P
	Control	DXR		Control	DXR	
12 h	42.8 ± 1.5	40.8 ± 2.5	n.s.	59.9 ± 6.0	58.8 ± 4.4	n.s.
3 days	48.1 ± 3.8	40.3 ± 3.8	< 0.01	56.9 ± 2.9	49.0 ± 3.1	< 0.01
6 days	30.1 ± 4.1	35.6 ± 2.4	n.s.	39.7 ± 4.9	41.4 ± 4.5	n.s.
4 weeks	43.6 ± 4.1	50.4 ± 4.2	n.s.	52.8 ± 2.8	50.1 ± 1.6	n.s.
5 weeks	36.8 ± 3.3	44.7 ± 3.9	< 0.05	49.6 ± 2.9	49.9 ± 5.4	n.s.

Values represent the mean ± SE of 5 experiments
Activity of cathepsin D is given as micrograms of tyrosine per hour per milligram of protein

Table 3. Effect of DXR (4 mg/kg IV weekly) on acid phosphatase total activity in mouse heart and liver

Time of treatment	Heart		P	Liver		P
	Control	DXR		Control	DXR	
12 h	0.70 ± 0.05	0.70 ± 0.07	n.s.	0.50 ± 0.09	0.50 ± 0.09	n.s.
3 days	0.82 ± 0.10	0.79 ± 0.09	n.s.	0.50 ± 0.07	0.56 ± 0.08	n.s.
6 days	0.60 ± 0.04	0.62 ± 0.20	n.s.	0.50 ± 0.10	0.50 ± 0.08	n.s.
4 weeks	0.81 ± 0.10	0.79 ± 0.20	n.s.	0.49 ± 0.09	0.46 ± 0.06	n.s.
5 weeks	0.67 ± 0.09	0.67 ± 0.06	n.s.	0.52 ± 0.10	0.41 ± 0.03	n.s.

Values represent the mean ± SE of 5 experiments
Activity of acid phosphate is given as micromoles of nitrophenol (NP) per hour per milligram of protein released from its substrate

Table 4. Effect of DXR (4 mg/kg IV weekly) on sedimentable enzyme activity of cathepsin D in mouse heart and liver

Time of treatment	Heart		P	Liver		P
	Control	DXR		Control	DXR	
12 h	57.2%±1.5	66.4%±2.3	<0.01	66.8%±1.4	66.6%±0.8	n.s.
3 days	58.6%±2.4	75.2%±1.5	<0.01	70.6%±1.3	76.8%±1.7	n.s.
6 days	58.4%±1.8	49.4%±2.6	<0.01	72.2%±1.7	69.0%±1.1	n.s.
4 weeks	58.8%±2.6	48.8%±4.4	<0.02	72.6%±1.2	67.2%±1.5	<0.02
5 weeks	58.6%±1.7	49.2%±4.2	<0.05	71.0%±1.3	67.4%±1.2	<0.02

Values represent the mean ± SE of 5 experiments and are expressed as the percentage of total [i.e., sedimentable (ML) + nonsedimentable (PLS)] enzyme activity that was sedimentable

Table 5. Effect of DXR (4 mg/Kg IV weekly) on sedimentable enzyme activity of acid phosphatase in mouse heart and liver.

Time of treatment	Heart		P	Liver		P
	Control	DXR		Control	DXR	
12 h	19.4%±1.7	20.8%±1.4	n.s.	58.2%±2.1	58.0%±2.1	n.s.
3 days	21.2%±1.7	23.4%±2.8	n.s.	61.0%±1.3	61.2%±1.3	n.s.
6 days	19.2%±1.6	16.6%±3.1	n.s.	61.0%±1.3	62.0%±2.5	n.s.
4 weeks	24.0%±3.1	20.4%±2.8	n.s.	60.8%±3.0	58.8%±2.4	n.s.
5 weeks	21.0%±1.9	23.2%±0.9	n.s.	58.0%±2.2	59.0%±1.8	n.s.

Values represent the mean ± SE of 5 experiments and are expressed as the percentage of total [i.e., sedimentable (ML) + nonsedimentable (PLS)] enzyme activity that was sedimentable

Table 6. Influence of DXR treatment (4 mg/kg IV weekly) on protein concentration in mouse heart and liver

Time of treatment	Heart		P	Liver		P
	Control	DXR		Control	DXR	
12 h	50.6±1.8	51.8±2.0	n.s.	114.8±5.3	106.9±6.0	n.s.
3 days	59.3±7.1	58.5±3.7	n.s.	130.0±3.1	123.7±3.2	n.s.
6 days	65.9±5.3	55.1±4.5	n.s.	122.0±5.8	118.0±3.4	n.s.
4 weeks	64.3±9.9	62.3±7.0	n.s.	132.9±8.1	131.2±8.8	n.s.
5 weeks	70.0±6.0	68.4±5.5	n.s.	128.8±5.8	130.2±6.8	n.s.

Values represent the mean ± SE of 5 experiments and are expressed as milligrams per gram of wet weight

ly in treated animals from the 12th hour after the first treatment onward and higher values persisted up to day 3; subsequently the cathepsin D sedimentability of the treated animals decreased to lower values than in the controls. In the liver a similar behavior was observed, but to a lesser extent.

Again, no modification of the sedimentability was observed in either organ for acid phosphatase (Table 5).

Finally, no differences in protein content were observed in either organ between treated animals and controls (Table 6).

Discussion

The present study demonstrates that in hearts of mice treated with DXR, both total enzyme activity and sedimentability of cathepsin D are markedly changed. These data agree with the hypothesis that lysosomes might play a role in anthracycline-induced chronic cardiotoxicity. On the other hand, these lysosomal changes could be nonspecific, i.e., they could be simply provoked by previous changes in cellular structures induced by the drug; in fact according to Morgan [17] and Ogunro [20], cathepsin D is probably the main hydrolase involved in physiologic and pathologic degradation of actomyosin, and we therefore need more research before we can come to a better understanding of the lysosomal enzymes role in DXR-induced cardiotoxicity.

Other studies however, reinforce the idea that lysosomes play the main role in DXR-induced cardiomyopathy: Mitochondria show a significant resistance to cathepsin D in vitro, but if the mitochondrial surface membrane

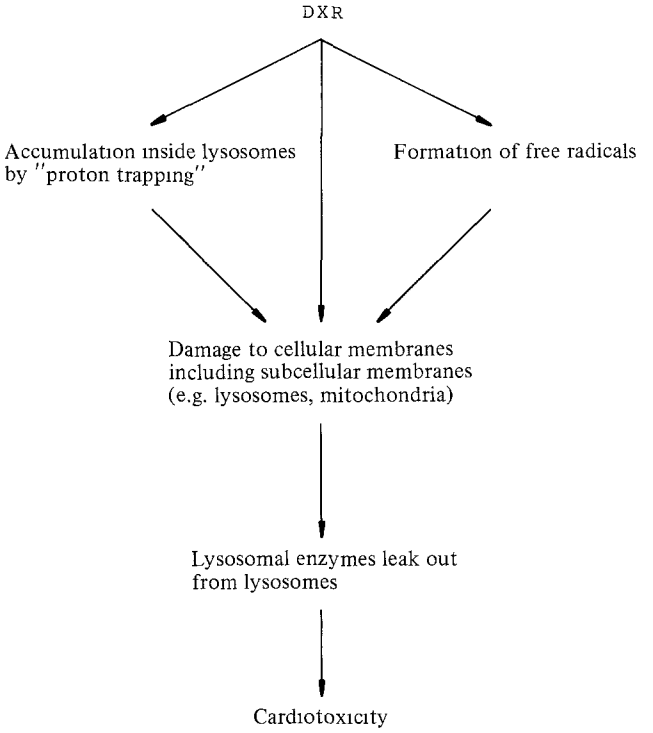

Fig. 1. Possible mechanism of lysosomal effects of DXR

has been even slightly damaged, marked hydrolysis of both structural and enzymatic proteins occurs [11]. In this connection, Duarte-Karim et al. [9], Murphree et al. [18], and Mikkelsen et al. [16] have demonstrated that DXR is able to damage cellular membranes even at low concentrations; furthermore, DXR is also able to cause free radical production, which in turn can injure cellular membranes [8, 24]. Moreover, Noel et al. [19] have shown in cultured rat embryo fibroblasts that DXR, and still more daunomycin, are actively accumulated reaching concentrations, inside lysosomes that are 3750 times and 20,000 times their extracellular concentrations, respectively, probably by way of proton trapping mechanism [7]. On the basis of these data we could then hypothesize that DXR-induced chronic cardiotoxicity depends on the sequence of effects shown in Fig. 1. It must also be stressed that the effects we have observed are much more marked in cardiac tissue especially those on total enzyme activity, a phenomenon which our data cannot explain but that is in good agreement with the cardiac specificity of DXR's cardiotoxicity with long-term administration. Furthermore, since the changes we have observed refer exclusively to cathepsin D and no modification was seen in acid phosphatase, it is conceivable that interstitial cells are unaffected or only very slightly affected.

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Received May 31, 1984/Accepted January 14, 1985