Effect of Adriamycin on heart mitochondrial DNA

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The effect of Adriamycin on rat heart mitochondrial DNA was determined by initially labelling the DNA with [*Me*-¹⁴C]thymidine. Animals subsequently received intravenously either Adriamycin (10 mg/kg) or saline (1 ml/kg) (control group). Animals later received [*Me*-³H]thymidine and were killed at 6, 14, 24 and 36 h after this. The heart mitochondrial DNA was separated into the linear, non-supercoiled and supercoiled forms by ultracentrifugation on formamide/sucrose sedimentation gradients, and the amount of ¹⁴C and ³H incorporated into the various forms of the DNA was determined by scintillation counting. The data suggest that Adriamycin may cause breakage of the mitochondrial DNA helix, and that it slows the rate of mitochondrial DNA synthesis and the formation of complete DNA molecules. These experiments show that Adriamycin does interact with heart mitochondrial DNA, and may explain the cardiac dysfunction association with Adriamycin use.

INTRODUCTION

Adriamycin has significant antitumour activity against a number of neoplastic diseases (Blum & Carter, 1974). The dose-dependent irreversible cardiotoxicity of Adriamycin is the major side effect which limits its use (Buzdar *et al.*, 1985; Haq *et al.*, 1985). Adriamycin cytotoxicity most closely correlates with nuclear DNA damage and inhibition of nuclear DNA replication and transcription (Potmesil *et al.*, 1984; Neidle & Sanderson, 1983; Schwartz, 1983). Various mechanisms have been proposed to explain the effect of Adriamycin. Although most evidence at present favours a direct effect of Adriamycin on nuclear DNA (Zunino *et al.*, 1979, 1980), there is evidence of Adriamycin-induced formation of oxygen free radicals as the mechanism of nuclear DNA damage and cytotoxicity (Lown *et al.*, 1977; Bachur *et al.*, 1982). However, neither mechanism explains the cardiomyopathy associated with Adriamycin.

Agents which bind to or damage nuclear DNA have the potential to bind to or damage mitochondrial DNA. In fact, some agents do so preferentially (Wunderlich *et al.*, 1970; Daugherty & Clapp, 1981; Backer & Weinstein, 1982; Niranjana *et al.*, 1982). The present study explores the effect of Adriamycin on mitochondrial DNA with regard to DNA synthesis and damage.

MATERIALS AND METHOD

Reagents

Sprague–Dawley female rats (225–250 g) were obtained from Southern Animal Farms. [*Me*-³H]Thymidine was obtained from New England Nuclear. Reagent-grade formamide and gradient-grade sucrose were obtained from Sigma.

Sedimentation on formamide/sucrose gradients

Sedimentation on formamide/sucrose gradients was performed as previously described (Ellis *et al.*, 1987). Briefly, two rats were stunned by a blow to the head and rapidly decapitated. The hearts were excised and finely minced in 0.25 M-sucrose/0.5 mM-EDTA/3 mM-Hepes, pH 7.2, at 4 °C. They were then homogenized together,

with a Dounce homogenizer. The homogenate was initially centrifuged at 600 *g* for 10 min to pellet the nuclei. This was repeated twice more, to ensure lack of nuclear DNA contamination. The final supernatant was centrifuged at 10000 *g* for 10 min to pellet the mitochondria. The mitochondrial pellet was washed twice in the isolation buffer. The final mitochondrial pellet was suspended in 0.15 M-sucrose/50 mM-KCl/25 mM-Tris, pH 7.4, at 4 °C. Linear formamide/sucrose gradients (5–15%, w/v), were formed with a two-chamber gradient mixer. A 0.5 ml portion of a lysis mixture [10 mM-NaCl/10 mM-EDTA/50 mM-Tris/20% (w/v) SDS] was placed on top of the gradient. Then 1 ml of the suspension of the intact mitochondria, containing approx. 5 mg of mitochondrial protein, was layered on to the gradient, and a final layer of 0.5 ml of the lysis mixture was added. The gradients were left for 2 h at 4 °C. Centrifugation was carried out in a Beckman SW 40 rotor at 140000 *g* for 2 h at 4 °C; then 0.5 ml fractions were collected dropwise on Whatman filter strips and allowed to dry overnight. After drying, they were immersed in three separate solutions of 10% trichloroacetic acid, 4 °C. After this, the strips were immersed in three solutions of 95% ethanol and allowed to air-dry. The strips were then cut into samples, and the amount of radioactivity was determined by scintillation counting.

RESULTS

Animals were injected with [*Me*-¹⁴C]thymidine (1 mCi/kg); 24 h later, they received Adriamycin (10 mg/kg) or saline (1 ml/kg), and 24 h after this, they received [*Me*-³H]thymidine (2 mCi/kg) (all injections intravenous). Animals were killed at 6, 14, 24 and 36 h after this last injection. Intact heart mitochondria were isolated and centrifuged on formamide/sucrose gradients as described above.

Fig. 1 shows a typical example of the graph obtained at each time point. The supercoiled mitochondrial DNA has the highest sedimentation coefficient (fractions 2–12). Intact circular mitochondrial DNA has an intermediate rate of sedimentation (fractions 14–24), and incomplete

Table 1. Total ^{14}C and ^3H incorporated into rat heart mitochondrial DNA

Average total ^{14}C and ^3H incorporated into rat heart mitochondrial DNA were measured in control and Adriamycin-treated rats at 6, 14, 24 and 36 h after injection of [$Me\text{-}^3\text{H}$]thymidine. Results (c.p.m.) are means $\pm$ s.d. ($n = 9$).

Time ...	6 h		14 h		24 h		36 h	
	Control	Adriamycin	Control	Adriamycin	Control	Adriamycin	Control	Adriamycin
^{14}C	16000 $\pm$ 1200	15300 $\pm$ 1700	16800 $\pm$ 1600	16300 $\pm$ 1400	15300 $\pm$ 1500	13300 $\pm$ 2200	16400 $\pm$ 2000	13900 $\pm$ 1700
^3H	6600 $\pm$ 900	3900 $\pm$ 1000	12900 $\pm$ 1100	4900 $\pm$ 800	13400 $\pm$ 1600	5400 $\pm$ 1200	13800 $\pm$ 1300	6300 $\pm$ 1300

mitochondrial DNA molecules or DNA molecules with breaks in the DNA helix are denatured to single strands of DNA and have the slowest rate of sedimentation (fractions 25–30) (Ellis *et al.*, 1987). The amounts of incorporated [$Me\text{-}^{14}\text{C}$]thymidine and [$Me\text{-}^3\text{H}$]thymidine were obtained by summing the total radioactivity (c.p.m.) in each peak.

The amount of incorporated [$Me\text{-}^{14}\text{C}$]thymidine was the same for Adriamycin-treated and control animals at all time points (Table 1). The amount of [$Me\text{-}^3\text{H}$]thymidine incorporated was markedly decreased for Adriamycin-treated animals at all time points (Table 1). The amount of [$Me\text{-}^{14}\text{C}$]thymidine incorporated into supercoiled DNA was the same for control and Adriamycin-treated animals. However, there was less [$Me\text{-}^{14}\text{C}$]thymidine incorporated in the intact circular non-supercoiled DNA, and a greater proportion in the linear form for Adriamycin-treated animals (Table 2).

The rate of incorporation of [$Me\text{-}^3\text{H}$]thymidine into supercoiled DNA is much slower for Adriamycin-treated animals than for controls (Table 3).

DISCUSSION

Mitochondrial DNA exists naturally in a negatively supercoiled form. The negative supercoiling is important in transcription and replication of the DNA (Cozzarelli, 1980). The product of replication of a mitochondrial DNA molecule is two intact circular non-supercoiled DNA molecules. At some point, superhelical turns are introduced into the molecule. Non-supercoiled intact circular mouse L-cell mitochondrial DNA has a lifetime of approx. 1 h before the introduction of superhelical turns. There is also a low frequency of intermediates between non-supercoiled and fully supercoiled molecules (Bogenhagen & Clayton, 1978).

Although there is no evidence of a DNA-repair phenomenon in animal mitochondria, there is evidence to suggest that damaged mitochondrial DNA is degraded *in vivo* (Clayton, 1982; Lansman & Clayton, 1975). The decreased proportion of [$Me\text{-}^{14}\text{C}$]thymidine in the non-supercoiled form of mitochondrial DNA, with an increase in the proportion in the linear form, suggests that Adriamycin causes breakage of the DNA helix of non-supercoiled mitochondrial DNA molecules, resulting in linear DNA. An alternative explanation is the conversion of the non-supercoiled DNA into supercoiled molecules, with decreased production of non-supercoiled molecules. The decreased rate of synthesis of non-supercoiled mitochondrial DNA molecules is shown by the slower rate of incorporation of [$Me\text{-}^3\text{H}$]thymidine into the non-supercoiled mitochondrial DNA fraction with Adriamycin.

These data support the hypothesis that Adriamycin reversibly intercalates between base pairs of mitochondrial DNA and terminates transcription of the DNA at that point. Mitochondrial DNA has only one origin of transcription. The product of transcription is a full-length complementary strand of RNA (Anderson *et al.*, 1981). Adriamycin inhibits completion of transcription, resulting in the formation of incomplete linear fragments of DNA.

At this dose, Adriamycin has been shown to inhibit both nuclear and mitochondrial DNA synthesis in liver and heart. This inhibition occurs for 2 days for liver

Fig. 1. Ultracentrifugation of rat heart mitochondria on formamide/sucrose gradients

The Figure shows an example of the data obtained from the ultracentrifugation of ¹⁴C- and ³H-labelled rat heart mitochondria from control and Adriamycin-treated animals on formamide/sucrose gradients at 6 h (a), 14 h (b), 24 h (c) and 36 h (d) after injection of [*Me*-³H]thymidine: ●, control, ¹⁴C; ○, control, ³H; ■, Adriamycin, ¹⁴C; ▲, Adriamycin, ³H.

Table 2. Effect of Adriamycin on the percentage of ¹⁴C-labelled DNA in the different forms of rat heart mitochondrial DNA

Average percentages (means ± s.d., *n* = 9) of the total incorporated ¹⁴C in the supercoiled, non-supercoiled and linear forms of rat heart mitochondrial DNA were measured at 6, 14, 24 and 36 h after injection of [*Me*-³H]thymidine.

DNA	6 h		14 h		24 h		36 h	
	Control	Adriamycin	Control	Adriamycin	Control	Adriamycin	Control	Adriamycin
Supercoiled	66.9 ± 2.4	66.9 ± 2.2	67.0 ± 2.2	66.7 ± 2.5	66.8 ± 1.7	66.3 ± 2.2	67.0 ± 1.8	66.3 ± 1.7
Non-supercoiled	17.0 ± 1.5	7.1 ± 1.5	16.8 ± 1.2	7.7 ± 1.0	16.9 ± 1.2	7.3 ± 1.0	17.6 ± 1.3	7.3 ± 1.2
Linear	16.1 ± 2.8	26.1 ± 2.5	16.2 ± 1.5	26.2 ± 1.7	16.3 ± 1.2	26.5 ± 1.7	15.4 ± 1.5	26.4 ± 1.4

nuclear, 4 days for heart nuclear, 8 days for liver mitochondrial and 12 days for heart mitochondrial DNA (Hixon *et al.*, 1981).

Adriamycin is particularly toxic to tissues with high energy demands met primarily by mitochondrial energy production. Interference with transcription of the

mitochondrial DNA may interfere with the production of mitochondrially encoded components of the oxidative-phosphorylation pathways and lead to diminished ATP production. This mechanism may be an important part of the cellular dysfunction and death associated with the use of Adriamycin.

Table 3. Effect of Adriamycin on the percentage of [*Me*-³H]thymidine incorporated into the different forms of rat heart mitochondrial DNA

Average percentages (means $\pm$ S.D., $n = 9$) of the total incorporated ³H in the supercoiled, non-supercoiled and linear forms of rat heart mitochondrial DNA were measured at 6, 14, 24 and 36 h after injection of [*Me*-³H]thymidine.

DNA	6 h		14 h		24 h		36 h	
	Control	Adriamycin	Control	Adriamycin	Control	Adriamycin	Control	Adriamycin
Supercoiled	0.7 $\pm$ 0.2	None	17.1 $\pm$ 1.4	None	63.8 $\pm$ 2.3	14.5 $\pm$ 1.0	67.5 $\pm$ 1.3	17.2 $\pm$ 1.2
Non-supercoiled	27.7 $\pm$ 1.9	None	40.8 $\pm$ 2.2	7.1 $\pm$ 1.3	18.2 $\pm$ 1.5	17.9 $\pm$ 1.3	17.5 $\pm$ 1.1	18.5 $\pm$ 1.5
Linear	71.6 $\pm$ 1.7	100 $\pm$ 0	42.2 $\pm$ 1.4	92.9 $\pm$ 1.3	18.0 $\pm$ 1.2	67.6 $\pm$ 1.2	14.9 $\pm$ 1.1	64.2 $\pm$ 1.9

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