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EFFECTS OF ADRIAMYCIN ON HEART AND SKELETAL MUSCLE CHROMATIN

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Abstract—Interactions between adriamycin (ADM) and chromatin from heart and skeletal muscle from 15 day-old chicken embryos were investigated. Adriamycin interacts with the DNA of chromatin and this interaction is modified by the chromatin proteins. One of the effects of this interaction is an increase in the melting temperature (T_m) of the DNA, where adriamycin is observed to increase the T_m of heart chromatin to a greater extent than skeletal muscle chromatin. Adriamycin also inhibits *in vitro* DNA and RNA synthesis in isolated chromatin and nuclei. This inhibition is observed to be greater in heart muscle. Inhibition of transcription in the myocardium could be a major cause of adriamycin-induced cardiomyopathy.

One of the anthracycline antibiotics used as a cancer chemotherapeutic agent is adriamycin. These drugs have diverse antineoplastic actions on tumours and commonly interact with DNA [1, 2]. The clinical use of adriamycin is limited by various toxic side-effects [3], such as cardiotoxicity, which can lead to congestive heart failure [4, 5]. Cardiac changes observed during adriamycin treatment include cessation of beating, degeneration of sarcoplasmic reticulum and mitochondria, and disarray of myofilaments [6–9].

The cardiotoxicity of ADM is likely to be multifactorial, since it has been shown to exert effects on many cellular functions, viz. an increase in lipid peroxidation and free radical formation [10], a decrease in metabolism of the heart [9, 11] by inhibition of the respiratory chain at the level of the mitochondrial enzymes [12, 13], and the transport of ions may also be affected [14]. The high affinity of ADM for cardiolipin would also explain the destruction of mitochondria in ADM treated hearts, since mitochondrial cardiolipin content is high [15]. In addition, it has been shown that drugs of anthracycline class may bind to DNA [16, 17] and cause a reduction in myocardial protein [18] and RNA content [19] in treatment animals.

Very little is known about the involvement or effects of the chromosomal proteins and their influence on adriamycin–DNA interaction. It has been shown that adriamycin can induce a major conformational change in chromatin and that this effect requires the presence of proteins associated with DNA [20].

The following paper deals with the interaction of adriamycin (ADM) with chromatin from heart and skeletal muscle and the effect on DNA and RNA synthesis of this interaction.

The results show a dose-dependent inhibition of DNA and RNA synthesis which is not identical in heart and skeletal muscle systems. The heart chromatin appears to be more susceptible to adriamycin-induced effects, which is presumably owing to the differences in template conformation, which is a function of nuclear protein content. The inhibition of RNA synthesis by direct interaction with chromatin–DNA is responsible for the observed decrease in myocardial RNA and protein content. These results show at least one of the mechanisms responsible for the development of adriamycin-cardiomyopathy. The understanding of the molecular basis of action could provide the basis for a better clinical prognosis for adriamycin-treated diseases.

MATERIALS AND METHODS

Preparation of muscle nuclei. Nuclei from 15 day-old chicken skeletal and heart muscle were prepared essentially as described by Koffer and Brownson [21] omitting Triton X-100 from buffers.

Preparation of chromatin for comparison of transcriptional activity using E. coli *polymerase (Method 1).* Nuclei were suspended and lysed in a buffer containing 80 mM NaCl, 24 mM EDTA, 1 mM sodium bisulfite, pH 6.0. The suspension was centrifuged at 10,000 *g* for 10 mins after which the pellet was resuspended and pelleted three times more in 0.15 M NaCl, 10 mM Tris–HCl, 1 mM sodium bisulfite, pH 7.5. The remaining pellet was stored at −80°.

Preparation of nuclear material for DNA and RNA synthesis with adriamycin (Method 2). Nuclei (0.5–1.0 g) were suspended in 3 ml Buffer I [1 mM Tris–HCl pH 8.0, 1 mM DTT, 1 mM PMSF (phenylmethylsulfonylfluoride), 1 mM EDTA] and then centrifuged at 15,000 *g* for 5 min at 20°. The pellet was resuspended and the nuclei lysed by a loose pestle in a Dounce glass homogenizer in Buffer II (Buffer I–PMSF). The suspension was centrifuged as before and resuspended in Buffer II (1 mM Tris–HCl pH 8.0, 1 mM DTT, 0,1 mM EDTA containing 40% v/v glycerol). The suspension was cen-

trifuged as before and the pellet finally suspended in 1–2 ml of Buffer II. This preparation has a high endogenous DNA polymerase activity [22].

In vitro *synthesis of DNA*. 15–20 μg of chromatin DNA (approximately 20 μl of solution) were suspended in 200 μl of buffer containing 50 mM Tris–HCl pH 7.5 mM DTT, 3 mM $MgCl_2$, 1 mM PEP (phosphoenolpyruvate) 8.5 units/ml PK (pyruvate kinase), 0.25 mM ATP, 0.1 mM dATP, dGPP, dTTP, and 0.02 mM [^{3}H]dTTP (sp. act. 49.2 Ci/mmole). Synthesis was allowed to proceed for 2 hr at 37°. Those samples to which adriamycin was added were incubated at 4° for 10 min prior to addition of [^{3}H]dTTP. Synthesis was measured by incorporation of acid-precipitable counts.

Transcriptional activity of chromatin using exogenous polymerase. To determine availability of chromatin to polymerase, exogenous (*E. coli*) polymerase was added. The incubation medium consisted of 200 μl of 80 mM Tris–HCl pH 7.8, 150 mM KCl, 12 mM $MgCl_2$, 4,8 mM dithiothreitol, 1 mM each of ATP, ATP and CTP, 0.05 mM [^{3}H]UTP (sp. act. 54 Ci/mmole), 2 units of *E. coli* K12 strain, DNA-dependent RNA polymerase (Miles Laboratories) and 5 μg of chromatin DNA. Incubation was at 37° for 1 hr after which reaction was terminated. Those samples to which adriamycin was added were incubated at 4° for 10 min prior to addition of [^{3}H]UTP. Synthesis was measured by incorporation of acid-precipitable counts.

Preparation of chick DNA. Erythrocyte chromatin, prepared as described previously, was suspended in 10 mM Tris–HCl pH 7.5 and pronase added to a concentration of 100 μg/ml. The solution was incubated at 37° for 24 hr, after which it was extracted twice with phenol. The aqueous phase was then dialysed against 50 mM Tris–HCl pH 7.5, 10 mM EDTA, 10 mM NaCl (200 vols.) for 4 hr and then 1000 vols. for 48 hr at 4°. The dialysate was adjusted to 0.5% SDS and pronase added as before. The solution was incubated at 37° for 12 hr and again twice phenol-extracted. The dialysis step, enzyme incubation and phenol extraction was repeated, after which the aqueous phase containing DNA was dialysed for 72 hr against three changes of 1000 vols. of 50 mM Tris–HCl pH 7.5, 10 mM EDTA, 10 mM NaCl at 4°. The DNA was subsequently lyophilized.

Fluorescent studies on adriamycin–DNA interaction. Adriamycin (0.5 mg/ml) in 10 mM Tris–HCl pH 8.0 was mixed directly with various concentrations of DNA (chromatin) and changes in intensity of fluorescence monitored in a Perkin–Elmer 204S spectrofluorometer (extinction 495 nm, emission 555 nm).

Quantitation of chromatin components. An aliquot (approximately 10 mg wet wt) of chromatin prepared by Method 1 was suspended in 10 mM Tris–HCl pH 7.9 and an equal volume of 10% TCA (trichloracetate) added. The solution was incubated at 100° for 20 min, followed by centrifugation at 10,000 *g* for 10 mins. The supernatant was assayed for DNA and the pellet for protein after resuspension in Tris–HCl as before. Protein was assayed by the procedure of Lowry *et al.* [23] and DNA by the diphenylamine method [24].

Thermal denaturation studies. Melting profiles of chromatin in 2.5×10^{-4} M EDTA pH 7.5 buffer were obtained from a Unicam SP1700 Ultraviolet spectrophotometer with a thermoregulated cell compartment and an Accuron SP876 temperature programme controller. Degassed solutions were placed in 1 ml Teflon stoppered cuvettes and the rate of heating was approximately 1° per min. The concentration of all samples was adjusted to an $O.D._{260}$ of 0.70.

DNA and RNA synthesis measured in intact nuclei. Nuclei were suspended in 25 mM Tris pH 7.4, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM EDTA and 20% glycerol and for DNA synthesis 20 μg DNA (nuclei) incubated in 200 μl of the following buffer: 25 mM Tris–HCl pH 8.8, 5 mM 2-mercaptoethanol, 5 mM EDTA, 40 mM KCl, 10 $MgCl_2$, 3 mM ATP, 0.04 mM dATP, dTTP, dGTP, 0.015 mM [^{3}H]dCTP (Sp. act. 49.2 Ci/mmole), 15% glycerol. Incubation was for 1 hr at 37°. RNA synthesis was done similarly; 40 μg DNA (nuclei) incubated in buffer as described above, but with ATP, GIP, CTP and [^{3}H]UTP.

RESULTS

The results of Fig. 1 show clearly that adriamycin (ADM) interacted with pure DNA and with chromatin DNA. In both cases, DNA was a powerful fluorescence quenching agent and interaction between ADM and DNA resulted in a decrease in intrinsic fluorescence of ADM. The change in the quenching effect between pure DNA and chromatin DNA shown in Fig. 1 illustrates the considerable influence that the chromosomal proteins have on this reaction, which is to reduce the quenching ability of the DNA. It was not possible to detect significant differences between the interactions of heart muscle and skeletal muscle chromatins with ADM using this method (Fig. 2). The similarities in fluorescence quenching are explained by the similarities

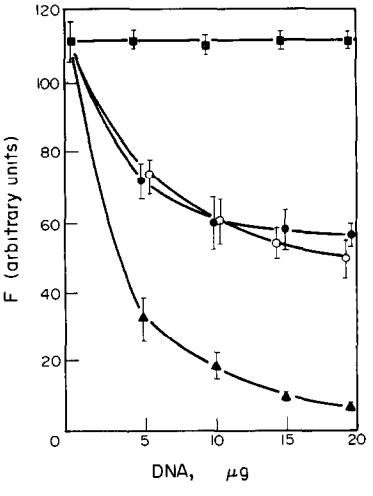

Fig. 1. Fluorescence behaviour of adriamycin. Adriamycin (5 μg) was titrated with increasing amounts of histones (■——■), pure DNA (▲——▲), heart muscle chromatin (●——●) and skeletal muscle chromatin (○——○). The fluorescence was monitored on a Perkin–Elmer 204S spectrofluorometer at excitation wavelength of 495 nm and emission wavelength 555 nm.

Table 1. Quantitation of chromatin components

Material	DNA	Histones	Non-histones
Skeletal muscle chromatin	1.00	1.17 ± 0.07	0.61 ± 0.05
Heart muscle chromatin	1.00	1.06 ± 0.04	0.80 ± 0.40

Standard deviations are shown.
$P < 0.005$ for histone and non-histone comparison.

of the total protein:DNA ratio in the two types of chromatin (Table 1).

The interaction between ADM and chromatin DNA was also investigated by means of thermal denaturation studies. The results show that the ADM–DNA interaction stabilized the double-helical chromatin DNA since the melting temperature (T_m) was increased (Table 2). The T_m of heart muscle chromatin DNA was increased on average slightly more than that of skeletal muscle chromatin.

However, the effects of adriamycin were most clearly seen at the level of gene activity. Figures 2–5 show the effect of ADM on transcription and DNA synthesis on isolated nuclei and chromatin from skeletal and heart muscle chromatin. In all situations, the synthesis of RNA and DNA was markedly decreased with an increase in ADM concentration which levelled off at higher concentrations of the drug. The effect was more marked in heart muscle preparations. The inhibition was dose-dependent (Figs. 2–5) and resulted in an 80% inhibition of DNA synthesis in isolated chromatin and approximately a 50% decrease in heart muscle nuclei and a 35% decrease in skeletal muscle nuclei (Figs. 2 and 3).

Isolated heart muscle chromatin showed greater sensitivity to ADM treatment to skeletal muscle chromatin only at low concentrations of ADM (Fig. 2), but in nuclei, this difference was maintained throughout the concentration range (Fig. 3), where inhibition of DNA synthesis was approximately 15–20% greater in heart muscle nuclei.

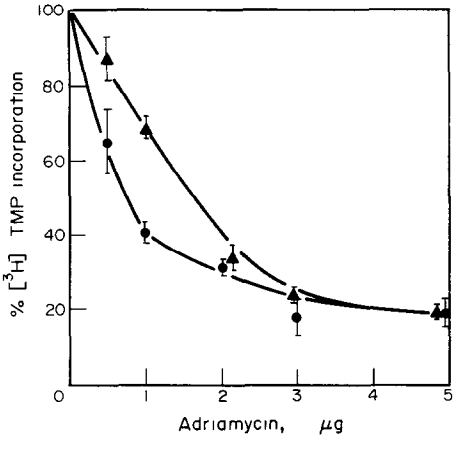

Fig. 2. Inhibiton of DNA synthesis in chromatin by ADM. Incubation contained 20 μg of chromatin DNA. Control [^{3}H]TMP incorporation; 600 dpm/μg DNA in skeletal muscle and 460 dpm/μg DNA in heart muscle. $P < 0.005$ up to 1 μg ADM and $P > 0.05$ for increased [ADM]. Heart muscle chromatin (●——●), skeletal muscle chromatin (▲——▲).

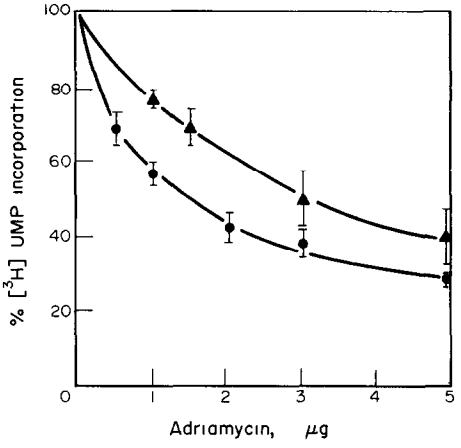

Fig. 3. Inhibition of RNA synthesis in chromatin by ADM (using *E. coli* polymerase). Incubation contained 20 μg of chromatin DNA. Control [^{3}H]UMP incorporation; 1750 dpm/μg DNA in skeletal muscle and 1230 dpm/μg in heart muscle. Average P estimate over the range of ADM concentrations; $P < 0.016$. Heart muscle chromatin (●——●), skeletal muscle chromatin (▲——▲).

Table 2. Thermal denaturation of chromatin

Material*	T_m(°)	T_m(°)(+ADM)†	ΔT_m(°)
Skeletal muscle chromatin	79.1 ± 0.22	82.4 ± 0.25	3.3
Heart muscle chromatin	79.9 ± 0.28	84.1 ± 0.24	4.2

* Chromatin prepared by Method 2.
† ADM:DNA (chromatin) 1:20 (w/w).
Standard deviations are shown for T_m values.
$P < 0.01$ for T_m and T_m + ADM.
$0.05 < P < 0.1$ for ΔT_m.

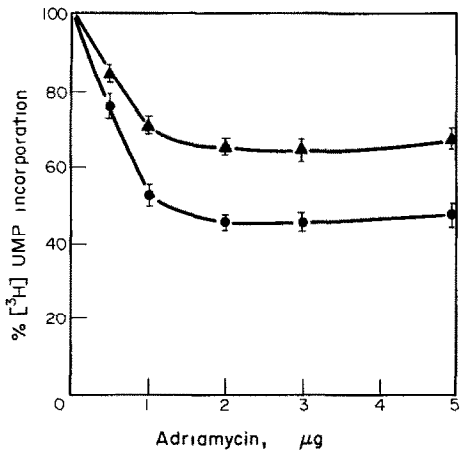

Fig. 4. Inhibition of DNA synthesis in nuclei by ADM. Incubations contained 20 μg of nuclear DNA. Control [^{3}H]TMP incorporation; 2744 dpm/μg DNA in skeletal muscle and 1885 dpm/μg in heart muscle. Average P estimate over the range of ADM concentrations; $P < 0.0016$. Heart muscle chromatin (●——●), skeletal muscle chromatin (▲——▲).

Similar differences in RNA synthesis were seen (Figs. 4 and 5), but in both chromatin and nuclei preparations, heart muscle material showed greater sensitivity throughout the concentration range. Heart muscle preparations showed a 10–20% lower rate of synthesis than skeletal muscle preparations where the inhibition was 70% in isolated heart muscle chromatin and 50% in nuclei. Similar results were obtained for isolated chromatin with endogenous (homologous) polymerase (results not shown).

DISCUSSION

The observed inhibition of DNA synthesis in muscle cell nuclei and isolated chromatin, showed that an interaction of the drug adriamycin with chromatin is responsible for the observed antimitotic nature of this drug *in vivo* [26]. Fluorescence and thermal denaturation studies showed that this interaction was with DNA and was modulated by chromosomal proteins.

The observed 80% decrease in DNA synthesis in chromatin and 60% in nuclei of heart muscle material is similar to that observed in cultured cardiac cells (75%) and the reduction observed *in vivo* [27, 28]. It is unlikely, however, that an inhibition of DNA synthesis by ADM would be responsible for ADM-induced cardiomyopathy, since adult cardiomyocytes have lost the capacity to undergo mitosis [29]. The reduction in DNA synthesis observed in adult hearts must therefore represent an inhibition of DNA synthesis in other cell types, such as endothelial cells, fibroblasts and neuronal cells. In embryonic hearts such as used in this study, this would not apply, since the cardiomyocytes actively undergo mitosis. The heart muscle material exhibited a lower genetic activity (DNA and RNA synthesis) than skeletal muscle and for this reason it seems likely that since active genes are exposed, ADM would bind preferentially to these regions of the DNA. Since heart material exhibits a lower activity, any given concentration of ADM is likely to affect heart material to a greater extent, as was observed, since there will be higher percentage occupation of available binding sites than in a relatively active genome (skeletal muscle). The inhibition of RNA synthesis was also observed to be greater in heart muscle material and the 50% inhibition observed in nuclei (heart) is in close agreement with a 52% decrease in myocardial RNA content in rats after ADM administration *in vivo* [19, 37].

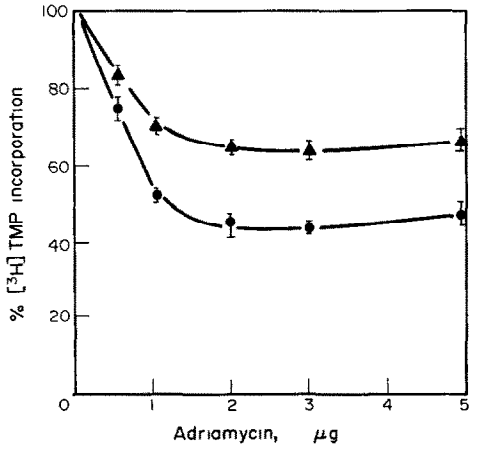

Fig 5. Inhibition of RNA synthesis in nuclei by ADM. Incubations contained 20 μg of nuclear DNA. Control [^{3}H]UMP incorporation; 494 dpm/μg DNA in skeletal muscle and 275 dpm/μg in heart muscle. Average P estimate over the range of ADM concentrations: $P < 0.001$. Heart muscle chromatin (●——●), skeletal muscle chromatin (▲——▲).

Similarities in inhibition of RNA and DNA synthesis (for both exogenous and endogenous polymerase activity) showed that the mechanism of action of ADM is not directly on the enzyme, but reacts with the substrate non-specifically (no recognition sites). This was confirmed by the T_m and fluorescence studies. The increase in T_m shown by chromatin on combination with ADM may explain in part the sensitivity of DNA to ADM treatment as measured by polymerase activity. Since polymerase act by 'melting' short regions of the DNA [30] any stabilization of the double helix would result in a decrease in polymerase activity. The larger increase in T_m of heart material may explain in part the greater inhibition of synthesis.

It follows that a decrease in RNA synthesis, must lead to a decrease in protein synthesis (as seen *in vivo* [18, 19, 37]), which may be the immediate cause of ADM-induced cardiomyopathy. Since heart muscle has an intrinsically low protein synthesis [31–33] and a high protein degradation rate [32] which maintains a critical balance, a decrease in protein synthesis due to inhibition of transcription could lead to a critical protein shortage which may lead to the disarray of myofilaments and cessation of beating observed *in vivo* [6–9].

Heart muscle would be especially susceptible to adriamycin-induced damage since the muscle cells are non-replicative (adult animals), only 25% of the cells are contractile and the rate of protein synthesis

is low whereas the rate of protein degradation is high. This can be compared to skeletal muscle which has regenerative (replicative) properties and where approximately 70% of the cells are contractile and have a relatively lower protein turnover rate [31–33].

Since the myocardium is dependent on aerobic metabolism and has a high metabolic rate, as well as a high rate of protein turnover, if either of these two processes should be interrupted, such as by ADM-treatment the myocardium will suffer damage. Owing to the genetically inactive state of the heart cell the damage may quickly lead to cell death. If the high susceptibility of the myocardium to ADM damage is coupled to a high affinity for ADM due to the presence of the phospholipids such as cardiolipin, [15, 34] it is understandable that ADM should affect the myocardium or any rapid-turnover cell in preference to a cell with a generally lower activity.

Since the proteolytic breakdown of proteins in the myocardium becomes crucially important during ADM-treatment, an inhibition of this process could possibly improve the clinical prognosis. Leupeptin, for example, has been found to decrease protein degradation in cardiac muscle *in vitro*, while having no effect on protein synthesis [35] and could therefore be useful in preventing atrophy. Leupeptin is nontoxic and is absorbed orally, but has the disadvantage of rapid excretion and a potential inhibition of important proteolytic enzymes, its disadvantages could perhaps be outweighed by potential advantages in the amelioration of adriamycin-induced cardiomyopathy. Antipain and pepstatin may be two other potentially useful agents.

Unlike its effect on cancer cells, where the antimitotic nature of ADM is of importance, it is probably the inhibition of RNA synthesis in cardiomyocytes which is a major cause of ADM-induced cardiomyopathy.

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